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UNIVERSITY OF ALBERTA

MOLECULAR CHARACTERIZATION OF DUPLICATIONS ASSOCIATED WITH CAT EYE SYNDROME

BY

ALAN J. MEARS ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Department of Genetics

Edmonton, Alberta
Spring, 1995

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This thesis is dedicated to the memory of my father Peter Jeffrey Mears, who in 18 years, gave me enough inspiration to last a lifetime.

Abstract

Cat eye syndrome (CES) is a rare human disorder. The phenotype is highly variable, but criteria for diagnosis include ocular coloboma, preauricular malformations, dysmorphic features, mild to moderate mental retardation, and defects of the heart, kidney and anus. This syndrome is the result of various types of duplications of chromosome region 22q11.2. Normally, a supernumerary dicentric marker chromosome is present, resulting in four copies of the region. However, interstitial duplications or supernumerary ring chromosomes have also been seen.

Using probes mapped to 22q11.2, extensive DNA dosage analysis combined with fluorescence *in situ* hybridization of metaphase chromosomes allowed determination of the size and structure of 22q11.2 duplications in 20 individuals. Characterization of the duplications enabled broad localization of the breakpoints associated with the formation of the duplications. It was observed that breakpoint heterogeneity occurred both within and between the groups with different types of duplications. This finding suggested that either distinct mechanisms could be involved in duplication formation or that multiple regions of 22q could act as the substrate for a single mechanism to act.

By comparing the phenotypes with the duplications, a phenotypic map for CES was derived. The critical region (CESCR) to which all phenotypic characteristics mapped, spanned a maximum of 2.1 Mb. Furthermore, the majority of characteristics, including the heart and kidney defects, mapped to a subregion of 1.0 Mb. Significantly, the CESCR mapped proximal to and did not overlap with the critical region of a deletion syndrome of 22q11.2, DiGeorge syndrome. This disproves a theory previously put forward by others that due to some phenotypic similarities shared by these two syndromes, the phenotypes represented the effects of deletions and duplications of the same genes in this region.

Comparison of the phenotypic effects of partial tetrasomy and partial trisomy of 22q11.2 suggested that although overall the phenotypic severity may reflect the level of dosage, a simple threshold model was insufficient to explain the wide variation in phenotype. This considered, the use of prenatal diagnosis to predict phenotypic outcome based on the level of dosage of the CESCR was determined to be highly problematic.

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I would like to gratefully acknowledge the following individuals who have influenced my research and life in Canada over the past five and a half years.

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Abbreviations

CE# = Patient designation number for case with interstitial duplication
CES = Cat eye syndrome
CESCR = Cat eye syndrome critical region
CM# = Patient designation number for case with supernumerary chromosome
CMT1A = Charcot-Marie-Tooth disease type 1A
CN# = Patient designation number for case with normal karyotype
CTAB = Cetyltrimethylammonium bromide
CTAF = Conotruncal face anomaly
DGS = DiGeorge syndrome
DGSCR = DiGeorge syndrome critical region
DMEM = Dulbecco's modified eagle medium
DNA = Deoxyribonucleic acid
DS = Down syndrome
DTAB = Dodecyltrimethylammonium bromide
EBV = Epstein barr virus
EDTA = Disodium ethylene diamine tetraacetate
FISH = Fluorescence in situ hybridization
ISCN = An International System for Cytogenetic Nomenclature
kb = Kilobase pairs of deoxyribonucleic acid
kDA = Kilodaltons
Mb = Megabase pairs of deoxyribonucleic acid
MEM = Minimal essential media
NCV = Nerve conduction velocity
NOR = Nucleolar organizing region
OD = Optical density
PBD = Phosphate-buffered detergent
PMP-22 = Peripheral myelin protein 22
RFLP = Restriction fragment length polymorphism
RNA = Ribonucleic acid
rRNA = Ribosomal ribonucleic acid
SDS = Sodium dodecyl sulfate
SR_D = Sum of ranks of disomic control data set
SRO = Smallest region of overlap
SR_P = Sum of ranks of patient data set

Abbreviations (continued)

SR_T = Sum of ranks of trisomic control data set

SSC = 150 mM sodium chloride/ 15 mM sodium citrate

TBS = Townes-Brocks syndrome

TE = 10 mM Tris pH 8.0/ 1 mM EDTA

VCFS = Velocardiofacial syndrome

YAC = Yeast artificial chromosome

Introduction

Chromosome 22 and associated anomalies

Human chromosome 22 is the second smallest autosome and accounts for approximately 1.8% of the human genome (Harnden and Klinger, ISCN 1985), which corresponds to about 54 Mb of DNA. The most recent estimate of the genetic map length is 94 cM (Genome Data Base and Cooperative Human Linkage Center, Johns Hopkins University School of Medicine, Baltimore, December 1994). Chromosome 22 is an acrocentric chromosome with a mostly heterochromatic p-arm made up of repetitive sequences. This p-arm varies significantly in size between individuals due to the presence of differing amounts of heterochromatin. The only known active genes in the p-arm are the rRNA genes, which are also present on four other acrocentric chromosome pairs (Kaplan et al. 1987). At the 400-band stage, the q-arm consists primarily of euchromatin which demonstrates two light G-bands (q11 and q13) separated by one dark band (q12). High resolution cytogenetic analysis (850-band stage) further subdivides these bands as shown in Figure 1. It has been predicted that there are approximately 1000-2000 genes on 22q of which less than 10% have been characterized according to Genome Data Base entries (December, 1994).

Chromosome 22 is associated with numerous chromosomal anomalies of both acquired and congenital origin. Cancer-related acquired anomalies include the t(11;22)(q24;q12) associated with Ewing's sarcoma (Aurias et al. 1983), the t(8;22)(q24;q11) associated with Burkitt's lymphoma (Berger et al. 1979) and the t(9;22)(q34;q11.2) associated with chronic myelogenous leukemia (Rowley et al. 1973) producing the so-called Philadelphia chromosome (Nowell et al. 1960). Deletions of 22q11-12 are associated with tumors of the central nervous system. These include meningioma (Deprèz 1991) and neurofibromatosis type 2 (Martuza and Eldridge 1988).

Chromosome 22 is also the site of numerous congenital chromosomal anomalies. Cat eye syndrome is the result of a duplication involving a small portion of chromosome 22. Cytogenetically, this syndrome is typically characterized by the presence of a supernumerary bisatellited marker chromosome derived from inverted duplications of 22pter-22q11.2. The (11;22) balanced translocation, which is the most common recurrent non-Robertsonian translocation in humans, results in reduced fertility and an abnormal phenotype in unbalanced offspring due to duplications of

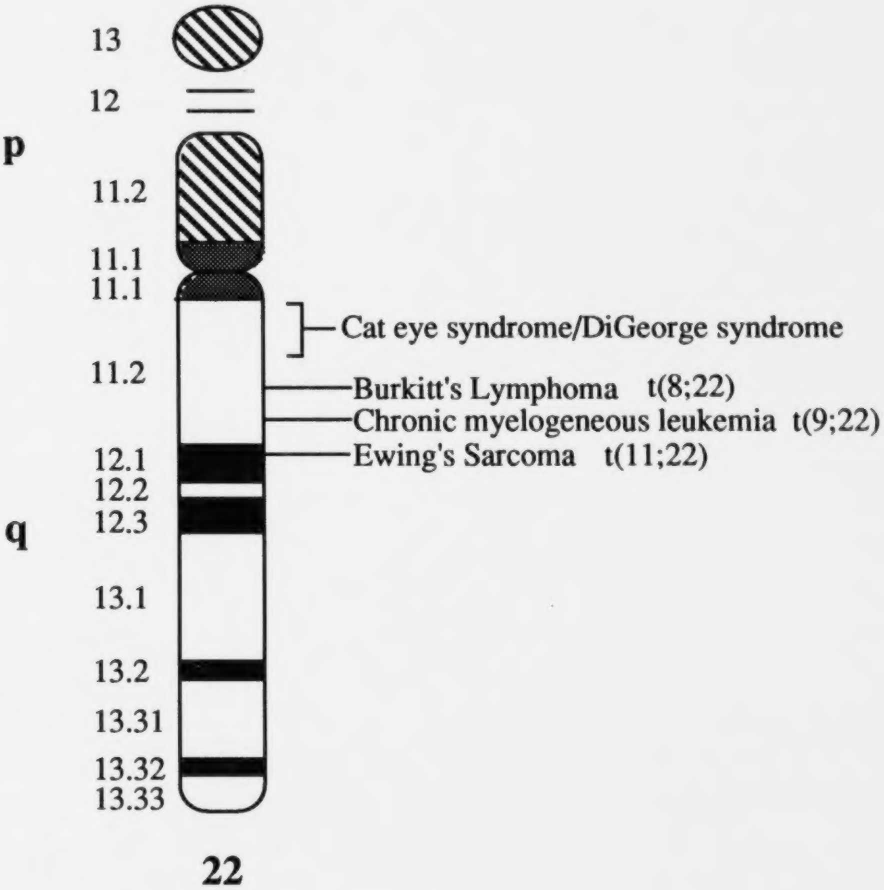

13
12
p
11.2
11.1
11.1
11.2
12.1
12.2
12.3
q
13.1
13.2
13.31
13.32
13.33
22
Cat eye syndrome/DiGeorge syndrome
Burkitt's Lymphoma t(8;22)
Chronic myelogeneous leukemia t(9;22)
Ewing's Sarcoma t(11;22)

Figure 1 Ideogram of chromosome 22 showing high resolution banding pattern (Harnden and Klinger, ISCN 1985). Approximate mapping of three cancer-related breakpoints, cat eye syndrome and DiGeorge syndrome is shown.

22q11.2 and 11q23.3. Full trisomy 22 is common (3-10%) in aborted fetuses (Hassold 1980; Lauritsen 1982) but rarely results in live-births, although several cases have been reported. DiGeorge syndrome, velocardiofacial syndrome and conotruncal face anomaly all involve similar deletions of 22q11. The distal portion of the long arm (22q13.3) is also associated with deletions (Nesslinger et al. 1994). Many of these congenital chromosomal anomalies are described in detail below.

Cat eye syndrome

Phenotypic Spectrum

The association of coloboma with anal atresia was first noted by Haab in 1878. Coloboma refers to defects associated with the iris, retina or choroid of the eye. These result from a failure of the optic cup to fuse by the ninth week of fetal development. If the defect involves the iris, the pupil appears "slit-like" (somewhat akin to the eyes of a cat) extending vertically into the lower part of the iris (Moore 1989). Anal atresia is the absence of an anal opening due to either the presence of a membraneous septum, which should normally rupture by the seventh week of fetal development, or complete absence of the anal canal (Moore 1989). In 1965, Schachenmann described the same association, but reported the cytogenetic finding of a small "abnormal" extra chromosome in four cases. By 1968, the term "cat eye syndrome" was used to describe this association (Gerald et al. 1968). The criteria for diagnosis was defined as (i) the presence of a small, sometimes bisatellited extra "marker" chromosome, (ii) mild or borderline mental retardation, and (iii) a pattern of congenital anomalies including anal atresia, ocular coloboma, hypertelorism, downslanting palpebral fissures, preauricular skin tags/pits, and cardiac and renal defects (Schinzel et al. 1981a). The only estimates for prevalence of CES are from Switzerland, where it is reported at a frequency in the range of 1 in 50,000 to 1 in 150,000 (Genome Data Base).

As realized by many investigators, although coloboma is relatively common in the reported cases of cat eye syndrome (CES), it is by no means an obligatory feature of this syndrome. Table 1 is a summary of the phenotypes from the reported cases of CES with the presence of a probable bisatellited marker chromosome. Many of these were previously summarized by Hsu and Hirschorn in 1977. From the collected data, the approximate frequencies of the phenotypic features are shown in Table 2 with the more common types of specific defects indicated in brackets.

Table 1 Phenotype summary of reported cases of CES with probable dicentric marker chromosome

No	PM	A A	CB	HD	RD	DP	HT	MR	ED	SD	GD	Reference
01	▨	▨		▨	▨	▨	▨	▨	▨	▨		Zellweger 1962
02			▨					▨	▨			Taft 1965
03	▨	▨			▨	▨	▨		▨			Ishmael 1965
04	▨	▨	▨		▨	▨						Schachenmann 1965
05	▨	▨	▨			▨						"
06		▨	▨		▨	▨		▨				"
07			▨		▨			▨				"
08	▨	▨								▨		Curcio 1967
09		▨		▨	▨			▨				Gerald 1968
10		▨			▨							"
11			▨	▨					▨	▨		Ginsberg 1968
12	▨	▨			▨		▨		▨	▨		Beyer 1968
13	▨	▨	▨		▨		▨	▨	▨			Thomas 1969
14	▨	▨	▨			▨	▨					Pfeiffer 1970
15	▨	▨	▨	▨	▨	▨	▨	▨	▨			Noël 1970
16	▨	▨	▨		▨			▨	▨			Weber 1970
17	▨	▨	▨									Darby 1971
18	▨					▨		▨				Krmpotic 1971
19	▨	▨		▨		▨						"
20	▨	▨	▨			▨	▨					Fryns 1972
21	▨					▨	▨	▨	▨	▨		Buhler 1972
22	▨	▨				▨	▨	▨		▨		"
23	▨	▨	▨	▨	▨			▨				Gerald 1972
24		▨		▨				▨				"
25	▨	▨										"
26	▨	▨	▨	▨	▨							"
27	▨	▨	▨	▨		▨		▨				Petit 1973
28	▨	▨		▨								Ballesta 1973
29	▨		▨		▨		▨					"
30	▨	▨	▨	▨	▨		▨					Cory 1974

Table 1 (continued)

No	PM	AA	CB	HD	RD	DP	HT	MR	ED	SD	GD	Reference
31	▨		▨	▨		▨	▨		▨	▨		De Chieri 1974
32	▨	▨	▨		▨		▨		▨			Kunze 1975
33	▨	▨	▨	▨	▨	▨			▨	▨		Pierson 1975
34	▨		▨		▨	▨	▨				▨	Noël 1976
35		▨		▨	▨	▨	▨			▨		"
36	▨	▨		▨				▨		▨		Bofinger 1977
37	▨	▨	▨									Weleber 1977
38	▨	▨	▨	▨			▨					Toomey 1977
39	▨	▨	▨			▨		▨			▨	Petit 1980
40	▨	▨	▨	▨	▨	▨						Schinzel 1981
41	▨	▨	▨	▨				▨				"
42		▨		▨	▨						▨	"
43	▨	▨	▨									"
44	▨	▨		▨		▨						"
45	▨	▨		▨		▨						"
46	▨											"
47	▨	▨		▨		▨			▨			Chemke 1983
48	▨	▨		▨		▨	▨					Wilson 1984
49	▨	▨				▨			▨			Rosenfeld 1984
50	▨	▨			▨	▨	▨		▨			Gabarrón 1985
51		▨	▨	▨					▨			Ing 1987
52	▨		▨	▨	▨	▨			▨			Magenis 1988
53			▨		▨	▨	▨		▨			Lüleci 1989
54	▨	▨	▨								▨	Ward 1989
55	▨											Cullen 1993
56	▨				▨							"
57	▨				▨				▨		▨	"
58	▨	▨		▨			▨					Urioste 1994
59	▨					▨	▨		▨			"

The table summarizes the phenotypic findings for 59 cases of CES reported in the literature. The 11 phenotypic characteristics scored are as follows:-

PM=preauricular malformations, AA=anal atresia, CB=coloboma,
HD=heart defect, RD=renal defect, DP=downslanting palpebral fissures,
HT=hypertelorism, MR=mental retardation, ED=ear defect (low set/rotated),
SD=skeletal defect, GD=genital defect

Shading indicates the presence of the characteristic.

Table 2

Summary of frequency of phenotypic characteristics reported for the cases in Table 1

Phenotype	**% freq**
Preauricular malformations	80%
Anal atresia (imperforate anus)	75%
Coloboma (of the iris)	50%
Downslanting palpebral fissures	50%
Heart defects (TOF, TAPVR)	45%
Renal defects (absent or hypoplastic kidney)	45%
Hypertelorism	35%
Low set/malformed ears	35%
Mental retardation (mild to moderate)	30%
Skeletal defects	15%
Genital defects (hypoplasia)	10%

Note: TOF = tetralogy of Fallot, TAPVR = total anomalous pulmonary venous return. % frequencies are calculated from the 59 cases listed in Table 1. The most common forms of defects are indicated in brackets.

In addition to the characteristic CES features of Table 2, gastrointestinal defects have been reported such as malrotated gut and Hirschsprung disease. Other dysmorphic features include inner epicanthic folds, flat nasal bridge and small mandible. It is important to note that the frequency approximates given in Table 2 are based on clinical reports and as such are biased. CES is usually diagnosed on the basis of clinical findings first followed by the cytogenetic analysis and identification of the CES marker chromosome. On this basis, it is highly likely that the cases that were brought to the attention of clinicians tended to involve the more severe or conspicuous phenotypic characteristics, especially coloboma. Mild cases may frequently be missed. In evidence of this fact, in 1971 two mothers of CES children were found to have marker chromosomes. One mother was clinically normal (Darby and Hughes 1971), the other had only preauricular malformations (Krmpotic 1971). The two mothers were only brought to the attention of the clinicians because of their children who demonstrated more severe CES phenotypes. This considered, it is likely that many of the serious and conspicuous congenital defects of CES are overestimated in frequency of occurrence. Another problem with the frequency estimates is the variability in detail of the clinical descriptions. Certain phenotypic characteristics may be omitted in the description such as dysmorphic features. Other features may be incompletely analyzed such as mental retardation, of which measurement is age and test dependent. With these characteristics, the frequencies may actually be underestimated.

Origins of the Marker Chromosome

The marker chromosome typically associated with CES is dicentric and bisatellited. The chromosomal origins of this marker chromosome evaded identification until the 1980's primarily due to its small size and lack of any distinct banding pattern with cytogenetic analysis. The presence of cytogenetic satellites at both ends indicated that the marker was derived from acrocentric chromosomes 13-15 or 22 (21 was considered unlikely at the time, due to the lack of the Down's Syndrome phenotype). In 1981, Schinzel described 11 CES patients with marker chromosomes (four of which had been previously described by Schachenmann in 1965). Extensive cytogenetic analysis including G, Q, C and R-banding was used to identify the marker but little conclusive information was derived from these methods other than the suggestion of chromosome 22 involvement in a few cases. Schinzel's conclusion that the markers were "probably" derived from chromosome 22 was primarily based on the similarities of the CES phenotype with the phenotypes of partial trisomy 22 and partial trisomy associated with the derivative (22) of the 11;22 translocation. Although the phenotypic comparisons were not highly correlative, the lack of virtually any phenotypic similarities with other acrocentric partial trisomies was considered significant. Thus Schinzel concluded that the bisatellited dicentric marker was probably derived from 22 and resulted in partial tetrasomy for 22q11 in these CES individuals. It wasn't until 1986 that the molecular evidence supporting the conclusions of Schinzel was provided. McDermid et al (1986) demonstrated by in situ hybridization that the 22q11.2-specific probe p22/34 (locus D22S9) was present on the bisatellited marker chromosome. Quantitative hybridization analysis from autoradiograms was used to determine the dosage of locus D22S9 in six CES individuals with marker chromosomes. All demonstrated four copies (tetrasomy) for the D22S9 locus. The results of these two studies verified the origins of the marker chromosome, and the typical karyotype for CES was then described as 47, +mar (inv dup 22pter-22q11.2) (Figure 2).

It is important to note that from cytogenetics alone, the marker chromosomes had been determined to vary in size when compared to the G group chromosomes (21, 22 and Y) as a standard (Mattei et al. 1984). One particularly large marker was described in the report of Rosenfeld (1984) in a patient with anal atresia, preauricular malformations, downslanting palpebral fissures and malformed ears. This patient was relatively mild in phenotype despite the size of the associated marker. However, no conclusive correlative studies between marker size and phenotype could be made with cytogenetic analysis alone.

Prior to the determination of the structure of the CES marker chromosome, similar structures had been reported for chromosome 15 (Schreck et al. 1977). These inv dup(15) marker chromosomes were readily identified because of their greater size and the ability to determine their origin using distamycin-DAPI banding (Schweizer et al. 1978). Several models were proposed to explain how the inv dup(15) dicentric marker chromosome was derived (Schreck et al. 1977; Van Dyke et al. 1977), and similar mechanisms may be involved in the formation of CES marker chromosomes. Of the models, two have been particularly favored in the literature.

The first model proposes the role of paracentric inversions (Srb and Owen 1952; Schreck et al. 1977). A crossover within the loop between the non-sister chromatids would result in one of the recombinant products being dicentric. It is usually assumed that the dicentric bridge would break during segregation. However, the fact that the dicentrics are often somatically stable is evidence that at least some of these markers can survive the rigors of division (Warburton et al. 1973; Van Dyke et al. 1977). It is proposed that inactivation of one of the centromeres may be the means by which such

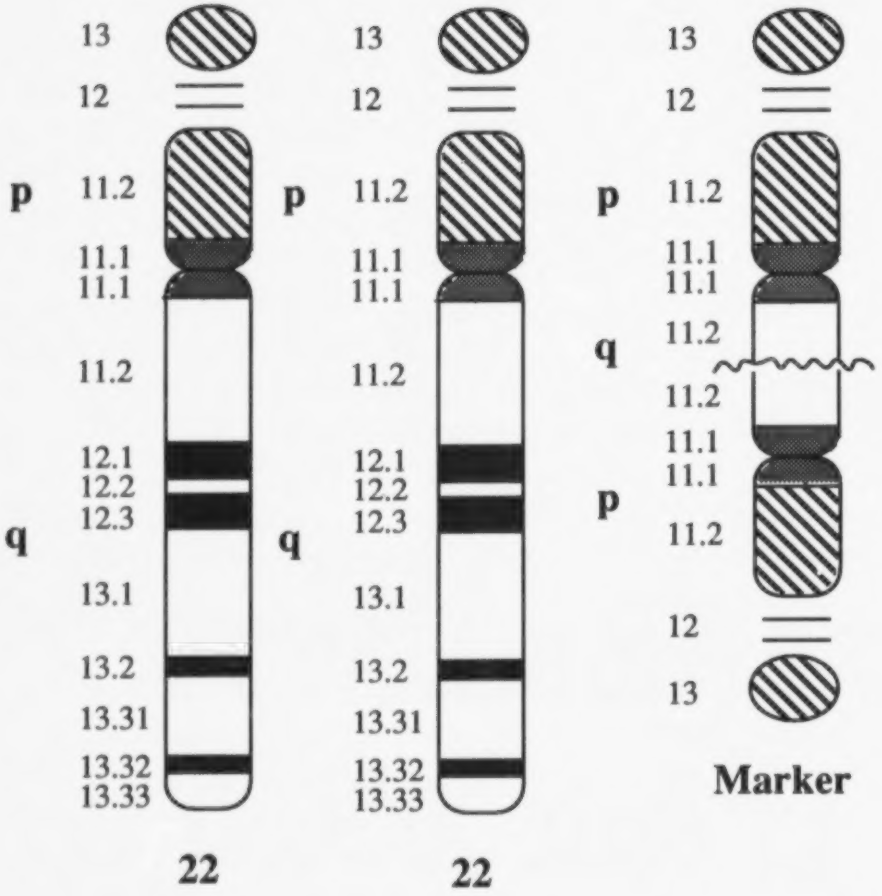

13
12
p
11.2
11.1
11.1
11.2
12.1
12.2
12.3
q
13.1
13.2
13.31
13.32
13.33
22
13
12
p
11.2
11.1
11.1
11.2
12.1
12.2
12.3
q
13.1
13.2
13.31
13.32
13.33
22
13
12
p
11.2
11.1
11.1
q
11.2
11.2
11.1
11.1
p
11.2
12
13
Marker

Figure 2 Ideogram of partial karyotype of a 47, + inv dup(22). The individual has two normal chromosomes 22 and a supernumerary marker chromosome. There is a total of four copies of the complete short arm region, and four copies of 22q11.1 and part of the 22q11.2 region. The jagged line indicates the break and fusion of the two inverted duplicated portions that comprise the marker chromosome.

stability is achieved (Schreck et al. 1977; Weleber et al. 1977). With only one active centromere a normal chromosome 22 and the dicentric marker may inadvertently segregate to one pole of the meiocyte (non-disjunction). Paracentric inversions have been identified in numerous autosomes (Fryns 1986; Del Porto 1984), but chromosome 22 does not lend itself well to cytogenetic detection of such rearrangements due to it's small size.

The second model to explain the derivation of the marker chromosomes proposes an illegitimate exchange event between normal sister chromatids or non-sister chromatids (Schreck et al. 1977; Van Dyke et al. 1977). Inverted repetitive sequences have been suggested as the potential medium by which such an exchange could generate inverted duplications (Mattei et al. 1984; Donlon et al. 1986; Robinson et al. 1993). As with the first model described, centromere inactivation and nondisjunction is again an essential component of this model.

Once a marker is formed, further rearrangements may occur. Van Dyke et al. (1977), Ing et al. (1987) and more recently Urioste et al. (1994) have reported the presence of several different types of secondary derivative structures such as smaller dicentric and monocentric fragments in individuals with dicentric marker chromosomes (derived from chromosome 15 or 22). It is assumed that such products are the result of instability of the marker chromosomes, possibly due to the marker chromosome having two active centromeres.

The parental origin of de novo marker chromosomes associated with CES has only been convincingly determined in two cases (Magenis et al. 1988). By cytogenetic analysis of chromosome 22 p-arm size variation, both ends of these markers demonstrated maternal origin, involving non-sister chromatids. Parental origin has also been reported in cases of inv dup(15), determined either by cytogenetic heteromorphisms (Wisniewski et al. 1979; Maraschio et al. 1981) or by RFLP's (Robinson et al. 1993). In nearly all cases (13/14), maternal origin has been demonstrated, and in these cases, non-sister chromatid exchange was either suspected or shown. In the one case of paternal origin, the marker chromosome was derived from a sister chromatid exchange (Maraschio et al. 1981). The preferential maternal origin observed in these cases is consistent with the models of marker formation that involve meiotic nondisjunction after exchange, as such an event has been shown to occur more frequently in females. Also consistent with previous data on such meiotic errors, an advanced maternal age is associated with cases of marker chromosomes of 15 (Wisniewski et al. 1979; Maraschio et al. 1981) and 22 (Schinzel et al. 1981a).

Marker chromosome and the CES phenotype

The net effect of the supernumerary bisatellited marker chromosome is that there will be an increase in dosage of the products of the genes within the duplicated region of 22pter-22q11.2. Some of these genes may be sensitive to such an increase, and the result is a disruption of their normal role, which manifests as the abnormal CES phenotype.

The only known active genes within the short arm of chromosome 22 are the rRNA genes (Kaplan et al. 1987), and it is assumed that there would be no phenotypic effect from change in dosage of these genes. Supporting evidence for this prediction is provided by other rearrangements of the acrocentric short arm. Firstly, (22;22) Robertsonian translocations, that result in loss of both p-arms have no phenotypic effect on the translocation carrier (Kirkels et al. 1980; Palmer et al. 1980) indicating that no essential functions have been lost. Secondly, small inv dup(15) chromosomes are often associated with a normal phenotype, (Cheng et al. 1994), which indicates that duplication of the rRNA genes alone is insufficient to result in a clinical phenotype. These lines of evidence suggest that the region of 22 responsible for the CES phenotype is 22q11.2 (22q11.1 is the centromere and should be devoid of genes). Within this duplicated region is the gene or genes responsible for the CES phenotype. Therefore, by definition, this region represents the preliminary "critical region" for the CES phenotype.

In cases of familial inheritance, the phenotype expressed by a CES individual with a marker chromosomes is apparently unaffected by its parental origin. Both paternal (Gerald et al. 1972; Noël et al. 1976; Cullen et al. 1993) and maternal inheritance (Schachenmann et al. 1965; Lüleci et al. 1989; Urioste et al. 1994) demonstrate the typical spectrum of CES characteristics. Thus, genomic imprinting of 22q11.2 has been considered unlikely as a contributory mechanism towards the variable expression of the CES phenotype. Direct evidence for the lack of maternal imprinting of chromosome 22 has been provided by three reports in the literature (Kirkels et al. 1980; Palmer et al. 1980; Schinzel et al. 1994). Both the 1980 reports describe familial inheritance of a (22;22) Robertsonian translocation from the carrier mother. The karyotypes of the normal offspring indicated loss of the paternal chromosome 22. Schinzel et al (1994) describe a de novo (22;22) Robertsonian translocation in a phenotypically normal male. Molecular analysis revealed that this rearranged chromosome is an isochromosome derived from one of the maternal chromosomes 22. These three reports all demonstrate normal phenotypes in individuals with no paternal contribution of chromosome 22. Thus it is concluded that there are no maternally imprinted genes with major phenotypic effects on chromosome 22.

Mosaicism of the Marker Chromosome

There is considerable phenotypic variation associated with CES, for which several possible explanations have been proposed. One possible contributory factor is mosaicism, which was noted in individuals with marker chromosomes in many reports of CES. Marker chromosomes, particularly dicentrics, may be unstable and lost from cells as they divide. This instability may be due to late replication caused by a high proportion of heterochromatin in the two p-arms, or breakage due to the presence of two active centromeres. The net effect of such instability is that specific cell lineages may not possess the marker chromosome in all the cells. The phenotypic effects of somatic mosaicism of marker chromosomes have been reported in several familial cases of CES using lymphocytes as the test tissue. The pedigrees of four such reports are shown in Figure 3 based on information of (a) Gerald et al (1972), (b) Urioste et al (1994), (c) Schachenmann et al (1965) and (d) Cullen et al (1993).

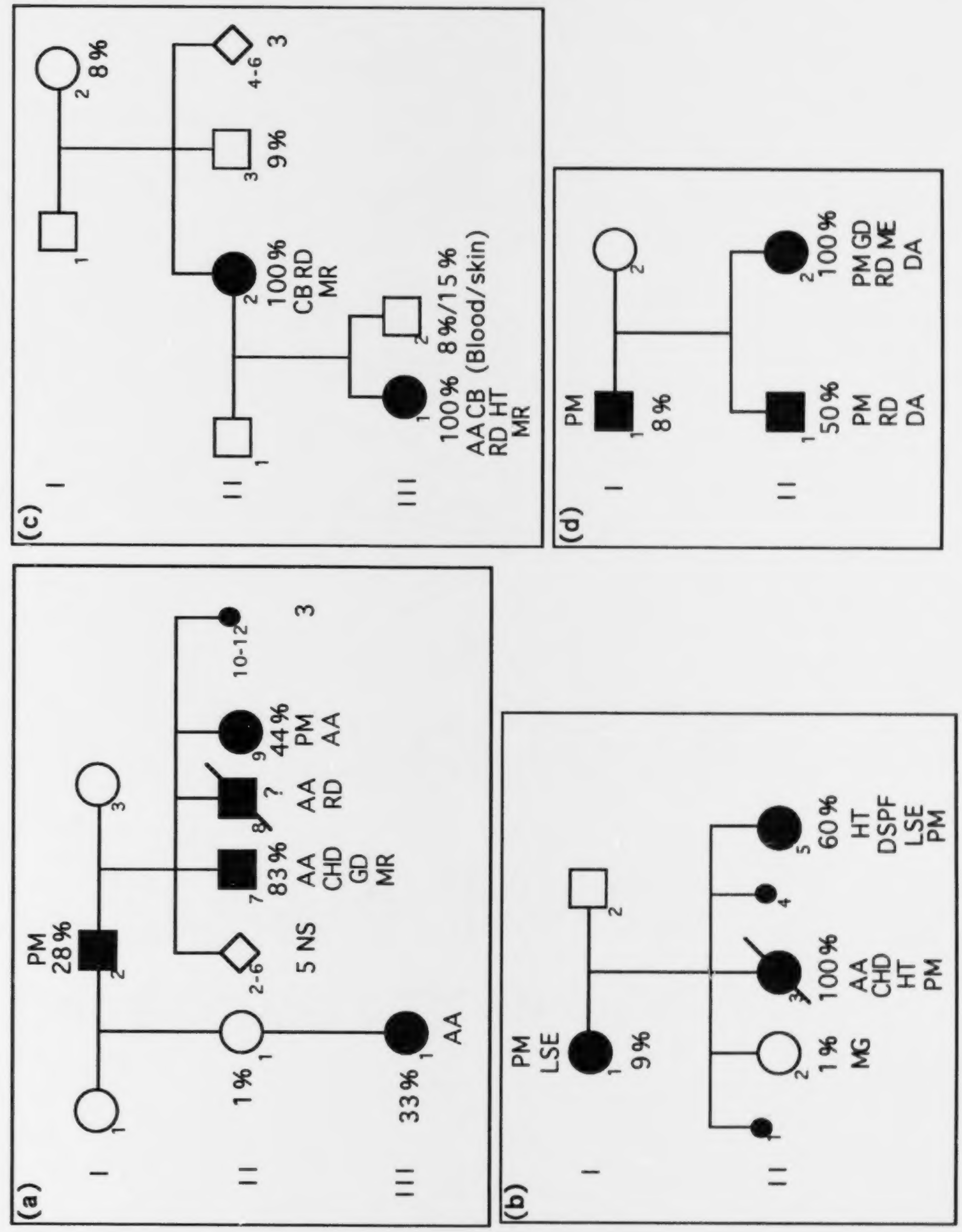
In pedigree (a), the father (I-2), who had only preauricular malformations, was 28% mosaic for the marker chromosome in his lymphocytes. Presumably the same marker was inherited by at least four of his children. One daughter (II-1) was only 1% mosaic and completely phenotypically normal. Her daughter (III-1) was 33% mosaic and had anal atresia. One son of I-2 (II-8) died from complications including anal atresia and renal defects (no cytogenetic studies were performed). Another son (II-7) demonstrated the marker in 83% of his cells and was more severely affected with a heart defect, congenital hearing loss, anal and genital defects and developmental delay. An affected daughter (II-9) had the marker in 44% of her cells but presented with only anal atresia and preauricular malformations. In this family there is a general correlation between percent mosaicism and phenotypic severity.

In pedigree (b), mosaicism was presented as a direct cause of the phenotypic variability (Urioste et al. 1994) The mother (I-1) only had the marker in 9% of her lymphocytes and had low set ears and preauricular malformations. The mosaic daughter (II-2) with only 1% of her cells containing the marker, was effectively normal with only micrognathia and myopia. Another daughter (II-5) showed the marker in 60% of cells and had hypertelorism, downslanting palpebral fissures, low set ears and preauricular malformations, but none of the "serious" congenital anomalies associated with CES. The daughter (II-3) with the marker in 100% of cells, died as a result of congenital

Figure 3 Familial cases of CES involving mosaicism of the marker chromosome.

Pedigree (a) is adapted from Gerald et al. (1972), (b) Urioste et al. (1994), (c) Schachenmann et al. (1965), and (d) Cullen et al. (1993). The numerical % refers to the number of cells (if scored) with the marker chromosome. (?) = unknown (not scored).

Phenotypic characteristics are reported as follows:-
PM = preauricular malformations, CHD = congenital heart defect,
AA = anal atresia, GD = genital defect, MR = mental retardation,
HT = hypertelorism, RD = renal defect, LSE = low set ears,
ME = malformed ears, MG = micrognathia, CB = coloboma,
DSPF = downslanting palpebral fissures, DA = Duane anomaly,
NS = no specific information



(a)
I
II
III
PM
28%
1
2
3
1%
1
2-6
5 NS
7
83%
AA
CHD
GD
MR
8
?
AA
RD
9
44%
PM
AA
10-12
3
1
33%
AA
(b)
I
II
PM
LSE
1
9%
2
1
2
1%
MG
3
100%
AA
CHD
HT
PM
4
5
60%
HT
DSPF
LSE
PM
(c)
I
II
III
1
2
8%
3
9%
4-6
3
1
2
100%
CB RD
MR
1
100%
AA CB
RD HT
MR
2
8%/15%
(Blood/skin)
(d)
I
II
PM
1
8%
2
1
50%
PM
RD
DA
2
100%
PM GD
RD ME
DA

anomalies. She presented with anal atresia, heart defect (total anomalous pulmonary venous return), hypertelorism and preauricular malformations. Of further interest in this family was the fact that cytogenetic studies revealed several distinct derivative structures that were assumed to be a result of breakage of the marker. These included small single satellite fragments and even tiny ring chromosomes.

Pedigree (c), represents some of the cases studied by Schachenmann's original report (Schachenmann et al. 1965). In this pedigree, the propositus (III-1) had the marker in 100% of her cells and showed a severe phenotype with coloboma, anal atresia, hypertelorism, renal defects and mental retardation. The mother of the propositus (II-2) also had the marker in 100% of her cells and had coloboma, mental retardation and renal defects but no anal abnormality. The maternal uncle (II-3) and grandmother (I-2) had the marker in only 9% and 8% of their cells respectively, but were phenotypically normal. Interestingly, the sibling (III-2) had the marker in 8% of his lymphocytes and 15% of the cells from a skin sample and showed a normal phenotype.

In pedigree (d) Cullen presented familial Duane anomaly (an unusual congenital strabismus) associated with a chromosome 22 derived bisatellited marker chromosome. The phenotypic characteristics of this family were somewhat unusual compared to many CES cases. The father (I-1) had the marker in 8% of his lymphocytes and had only preauricular malformations. The son (II-1) demonstrated the marker in 50% of his cells and presented with preauricular malformations, congenital hearing loss, a renal defect and Duane anomaly. The daughter (II-2) had the marker in 100% of her cells studied and had preauricular malformations, a renal defect, a genital defect, malformed ears with hearing loss and Duane anomaly. Note that neither coloboma or anal atresia was observed with this family.

These familial studies of mosaicism and CES raise several important questions. One criticism of the measurement of mosaicism is that of the limitations of cell type that may be tested. The presence of the marker chromosome is typically determined from one cell type, usually the lymphocytes. Rarely is a second cell type (skin) studied. In the case of individual III.2 of pedigree (c), the level of mosaicism was determined to be different between the two cell types with the marker present in 8% of lymphocytes compared to 15% of the skin cells. Whether such a difference is significant is difficult to determine. This finding leads to the question of whether the level of mosaicism determined from one cell type is representative of all the somatic cells. Differences in mosaicism between different tissues may be a reflection of different tissue-specific stringency's placed upon the marker that may incur its loss. Indeed, if tissue-specific variable mosaicism is occurring, this may in itself be a major contributory factor towards the specific phenotypic characteristics that are observed in a patient.

Another consideration for studies of mosaicism is the timing of the loss of the marker chromosome. Over the early developmental period the marker may be progressively lost from cells, such that by the time an adult sample is taken, very few cells contain the marker. The congenital anomalies occur in the developing fetus and the level of mosaicism in this window of time would be the critical factor. In case of point, some of the pedigrees described demonstrate situations whereby parents with very low percentages of the marker in their cells, have a disproportionate larger number of offspring with the marker than would be predicted. In pedigree (b) of Figure 3, the parent has the marker chromosome (I-1) in only 9% of cells, but two of three liveborn children also have the marker chromosome. This observation tentatively suggests that the marker is present in a much larger proportion of the cells of the germline than would be indicated by the level of mosaicism determined from the lymphocytes of the adults.

The pedigrees of Figure 3 indicate the transient and variable instability of the marker chromosomes that may be associated with CES pedigrees. In all pedigrees presented, the same marker, apparently unstable in one individual, may become more stable in the offspring. The mechanistic basis by which this is achieved is intriguing. It is possible that the second centromere of the marker chromosome may activate and inactivate as it is passes through the germline and through different cell types during division and differentiation. In these four pedigrees, if it is assumed that the measured mosaicism in lymphocytes is at least a general reflection of the stability of the marker in individuals, and that marker is assumed to be the same (no further rearrangement) through the generations, then the evidence would suggest that loss of the marker chromosome contributes to a less severe phenotype. To counter the proposed phenotype/mosaicism correlation model, the role of other factors in the variability of phenotype is apparent in numerous pedigrees. In pedigree (c), the propositus and mother displayed different phenotypes even though the marker is in 100% of the lymphocytes analyzed cytogenetically. Noël (1976) presented a non-mosaic father and son, with the father having coloboma and preauricular malformations and the son presenting with anal atresia and a heart defect. A more dramatic example was presented by Lüleci (1989) in which the mother and sister of the severely affected propositus were phenotypically normal despite the presence of the same marker in 100% of cells examined. From the literature, therefore, there is evidence of both stability and instability of these marker chromosomes, both associated with phenotypic variability. With the information available, it is apparent that the variable instability of the marker chromosomes may play a role in the expression of the CES phenotype. However, lack of ability to measure this phenomenon over time and in different tissues of a developing affected fetus prevent any concrete conclusions on a direct correlation between the loss of the marker and the phenotypic severity of CES being formulated.

Interstitial duplications and cat eye syndrome

CES is assumed to be caused by the dosage effect of having four copies of a gene or genes within 22q11.2. One possible contributory factor towards phenotypic variability is the dosage of the genes in the region. Two publications (Reiss et al. 1985; Knoll et al. 1995), describe patients with interstitial duplications of 22q11 and therefore only 3 copies of 22q11.2. LW (Reiss et al. 1985) had colobomata, preauricular pits, hypertelorism, downslanting palpebral fissures and developmental delay. SK (Knoll et al. 1994) presented with hypertelorism, downslanting palpebral fissures, preauricular pits, total anomalous pulmonary venous return, congenital hearing loss, absent right kidney and testicle and moderate motor delay. These patients were cytogenetically shown to have interstitial duplications of the CES region (22q11), demonstrating three copies of this region. LW was described as 46, XY, dup (22)(pter-q11.2::q11.1-qter) and SK as 46, XY, dup(22)(pter-q12::q11.2-qter). Molecular analysis of LW was reported by McDermid et al (1986) where the D22S9 locus was demonstrated to be in three copies. For SK, Knoll et al. (1994) demonstrated three copies for the IGLC locus, which is distal to the Burkitt Lymphoma breakpoint (Figure 1). The significance of these patients is that between them, all the major phenotypic characteristics of CES were represented except anal atresia. Thus, three copies of 22q11.2 was sufficient to cause features of CES. However, as with marker chromosome CES reports, sampling bias for such severe cases probably misrepresents the frequency of the more severe phenotypic characteristics of CES. Less severely compromised or normal individuals with interstitial duplications are unlikely to be detected in the populace. It is assumed that mosaicism was not a contributory factor towards the phenotypic variability of these patients. The size of the region duplicated in these individuals may differ and produce the phenotypic variability observed in these patients.

Cat eye syndrome with normal karyotype

The majority of the CES cases reported have demonstrated the presence of the marker chromosome. There are a few reported cases of apparent CES with a normal chromosomal constitution. The phenotypes of these individuals are presented in Table 3. From this table, on phenotype alone, the diagnosis of CES is likely, with all four individuals demonstrating at least two of the major phenotypic features of CES (coloboma, anal atresia and preauricular malformations). The patients described by Franklin and Parslow (1972) are sisters. If it is assumed that these are cases of CES, several possibilities exist:-

(1) *complete loss of the marker*, whereby a CES marker chromosome was originally present but has been completely lost from the cell type on which karyotyping was performed.
(2) *interstitial duplication* , whereby a cytogenetically undetectable (less than 3-4 megabases) duplication exists in 22q11.2.

Such patients may prove to be a key resource for the identification of the CES critical region, however none of these patients are available for this study.

However, care must be taken in assigning a diagnosis of CES in the absence of a marker chromosome or clear interstitial duplication. There are many examples in the literature of syndromes with different etiologies sharing many common phenotypic characteristics. This is probably due to a common defect resulting from different perturbations of the same developmental pathway. Mental retardation and congenital heart disease represent two characteristics of CES which are observed in many other aneuploidy syndromes, probably reflecting the complexity of the development of the heart and brain. All the phenotypic features of CES are seen as parts of other syndromes or as isolated defects. A pattern of phenotypic features is necessary for the identification of a given syndrome, but due to the phenotypic variability that is often observed misdiagnosis may occur, especially in cases of syndromes that share many features. For instance, Townes-Brocks syndrome (TBS) may be phenotypically characterized by preauricular pits and tags, sensorineural hearing loss, dysplastic ears, imperforate anus or anal stenosis, numerous thumb, finger and toe anomalies, hypoplastic kidney, and a heart defect (ventricular-septal defect)(Townes and Brocks 1972; O'Callaghan and Young 1990). The phenotypic similarities with CES is striking, but this autosomal dominant disease is not due to obvious aneuploidy and is believed to

Table 3

Summary of literature cases of apparent CES with normal karyotypes

Phenotypic Characteristic	Zellweger (1962)	Neu (1970)	Franklin (1972)	
Coloboma	+	+		+
Anal atresia	+	+	+	+
Preauricular malformations			+	+
Downslanting palpebral fissures		+		
Hypertelorism		+	+	+
Renal Defects		+		
Congenital Heart Defect	+	+	+	+
Mental Retardation	+		+	+
Skeletal Defects		+	+	+
Low set or malformed ears	+	+	+	+

Note.- a plus sign indicates that the phenotypic characteristic was reported in the study. Heart defects included tetralogy of Fallot and septal defects.

be linked to chromosome 16p12.1 (Friedman et al. 1987; Serville et al. 1993). The phenotypic variability observed within TBS makes differentiation from CES using phenotype even more problematic. Another putative syndrome, that has been tentatively localized to chromosome 2, demonstrates iris coloboma, ptosis, hypertelorism and mental retardation as its common phenotypic features (Pallotta 1991), again showing overlap with CES. Thus the presence of the marker chromosome remains the cardinal feature distinguishing CES from other syndromes.

Diagnostic complications aside, such similarity between syndromes raises interesting questions on the nature of the genes involved. Though the organs and systems affected may just be demonstrating a limited repertoire of anomalies, it is possible that the associated genes may share functional roles or indeed even be part of a homologous family of developmental genes. The apparent genetic heterogeneity of certain CES phenotypic characteristics may be an example of such homologous genes associated with general or specific systemic development.

Other syndromes associated with 22q11.2

Trisomy 22

An abnormality related to CES is that of trisomy 22. As a duplication of the entire chromosome 22, direct comparisons to the CES phenotype can be made and similarities are expected. Trisomy 22 is relatively common in spontaneous abortions, with estimates from 3% (Hassold 1980) to as high as 10% (Lauritsen 1982). However, it is very rare in liveborn infants. In the 1977 review by Hsu and Hirschorn, 19 "confirmed" cases of trisomy 22 were reported. However, Schinzel et al. (1981b, 1981c) credits only three probable cases by high resolution banding. The others represent cases of partial trisomy 22 derived from a variety of unbalanced products of translocations including the (11;22) translocation. The phenotypic findings of eight more recent confirmed cases of trisomy 22 are used for a phenotypic comparison with CES, and are shown in Table 4 (Kobrynski et al. 1993; Iselius & Faxelius 1978; Peterson et al. 1987; Voiculescum et al. 1987; Kukolich et al. 1989; McPherson et al. 1990; Sundereshan et al. 1990; Stratton et al. 1993).

As expected, many of the CES phenotypic features are present within the phenotypic spectrum of the trisomy 22 cases. Facial dysmorphism, ear anomalies, heart, renal and anal defects are all well represented amongst these 8 cases. The low level of mental retardation reported (1/8) is due to early neonatal death precluding any such

Table 4

Frequency of phenotypic characteristics observed in 8 confirmed trisomy 22 cases

Phenotypic characteristic	Frequency
Mental retardation	1/8
Broad flat nose	5/8
Hypertelorism	5/8
Long philtrum	1/8
Downslanting palpebral fissures	2/8
Micrognathia	5/8
Hypotonia	3/8
Ear anomalies	7/8
Small kidneys	3/8
Heart Defect	6/8
Genital hypoplasia	3/8
Anal atresia	3/8

Note.- Heart defects included tetralogy of Fallot. Other features commonly noted but not presented in the table include cleft lip/palate, webbed neck, and skeletal anomalies.

measurement of the level of development. One characteristic conspicuous by its absence is that of coloboma. In none of the confirmed eight cases of trisomy 22 has coloboma been noted. It is important to note that the phenotypes associated with trisomy 22 are much more severe than those of CES. This is reflected by the high level of spontaneous abortion, and of those fetuses that survive to term, a high level of neonatal mortality. Hence, these cases of confirmed trisomy 22 are highly selected cases that may reflect the "mild" end of the phenotypic spectrum. The interstitial duplications have already provided evidence that three copies of 22q11 is sufficient to cause the CES phenotype including coloboma (Reiss et al. 1985). The differences may lie in the complex biology of the states of activation and interferences placed upon the developmental pathways by the presence of a complete extra chromosome compared to the presence of duplications of a particular small region of 22q11.2. Indeed, the viability of trisomy 22s is very low compared to that of CES, indicative of the additional developmental "stress" caused by the extra duplicated material of chromosome 22.

11q;22q translocation

Duplication of 22q11.2 is also seen in the form of an unbalanced product of the (11;22) translocation. As with trisomy 22, comparisons can be made between the phenotypes of CES and the unbalanced offspring of this translocation.

The t(11;22) is the most common non-Robertsonian translocation in humans (Fraccaro et al. 1980; Iselius et al. 1983). Balanced translocation carriers are phenotypically normal, but of the possible unbalanced offspring that may result, only the 3:1 segregation product is viable [karyotype: 47 XX or XY, + der (22), t(11;22)(q23.3;q11.2)]. Due to this fact, reduced fertility is observed in carriers of this reciprocal translocation (especially males) and spontaneous abortions are common. Of the liveborn offspring, only 2-3% are unbalanced . As a result of the 3:1 disjunction, typically in the mother, these offspring are duplicated (partially trisomic) for 22pter-22q11.2 and 11q23.3-qter. The resulting phenotypes of these offspring are highly variable but from 47 cases (Fraccaro et al. 1980), 80% had malformed ears or preauricular malformations, 85% exhibited developmental delay, 70% skeletal defects, 50% genital defects, and 40% congenital heart defects (usually septal defects). Anal atresia, renal defects and typical CES dysmorphic features were only evident in 20% of cases or less. Numerous additional phenotypic anomalies were noted, many of which overlapped with those of trisomy 22, such as cleft palate, craniofacial asymmetry, large nose, microcephaly, dislocated hip joints, hypotonia, strabismus and skin anomalies. In general, especially due to the severe developmental delay that may be exhibited in the unbalanced offspring, the prognosis is very poor.

Interestingly, there is only one reported case of coloboma associated with this translocation (Simi et al. 1992), in which a boy [47, XY, t(11;22) (q23.3;q11.2),+ der (22) t(11;22) (q23.3;q11.2)pat] demonstrated micrognathia, microcephaly, craniofacial asymmetry, low set ears with preauricular tags, strabismus, iris coloboma and ectopic anus. It is curious that coloboma is seen so rarely (less than 2% of reported cases) with the partial trisomy of 22q11.2 of these patients, and has not yet been observed in trisomy 22. It is possible that additional gene products resulting from duplicated material distal to the region duplicated in CES may interfere with the aberrant developmental processes that lead to coloboma. By cytogenetics, the 22q11.2 duplication in the t(11;22) is not excessively larger than that of the CES duplications, which implicates gene(s) of 22q11.2 in this possible inhibitory process. The one case of coloboma may be explained by the duplication of 22q11.2 in this case being smaller than in the majority of cases, or genetic background and chance may be major contributory factors in this rare phenotypic finding.

Deletion syndromes of 22q11.2

Cytogenetic band 22q11.2 is the location of three haploinsufficiency syndromes: DiGeorge (DGS), velocardiofacial (VCFS) and conotruncal anomaly face (CTAF). DGS is typically associated with congenital heart defects, (usually of the conotruncal variety such as interrupted aortic arch or tetralogy of fallot), hypocalcemia, small or absent thymus and some facial dysmorphism (DiGeorge 1965). VCFS has conotruncal cardiovascular anomalies, cleft palate, behavioral problems and facial dysmorphic features including a prominent nose and narrow palpebral fissures (Sphrintzen et al. 1981). CTAF shows dysmorphic features of which some are common to the other two syndromes, minor ear anomalies and conotruncal heart defects (Kinouchi et al. 1976). Despite being considered clinically distinct entities, the three syndromes show considerable phenotypic overlap, particularly in their cardiac defects.

Of the three, DGS is the most studied at the molecular level. DGS is a developmental field defect of the III-IV pharyngeal pouches. It has a highly variable phenotype and has several possible distinct etiologies. The most common etiology for DGS was discovered to be partial monosomy of 22q11.2. About 10-15% of DGS cases demonstrated cytogenetically detectable deletions of 22q11.2, associated with translocations (de la Chapelle et al. 1981; Kelley et al. 1982; Greenberg et al.

1984,1988) or interstitial deletions (Greenberg et al. 1988; Mascarello et al. 1989). Molecular analysis discovered that cytogenetically undetectable deletions (microdeletions) of less than 4 Mb were the more common etiological cause (Driscoll et al. 1992a; Carey et al. 1992). In studies of diagnosed DGS cases, nearly 90% are found to have deletions of the same region. These deletions were subsequently mapped at high resolution to determine the smallest region of overlap (SRO) (region deleted in all cases), which would represent the critical region of DGS (DGSCR). The DGSCR has been shown to span three loci; D22S75-D22S66-D22S259 (Figure 4). Estimates for the size of the critical region is in the 500-750 kb range (Goldmuntz et al. 1993; Halford et al. 1993b).

Deletions have also been detected in the majority of VCFS and CTAF cases and these deletions show considerable overlap with those of DGS (Driscoll et al. 1993; Burn et al. 1993; Matsuoka et al. 1994). In the few cases in which no deletions were found, smaller deletions (between the identified loci) or point mutations in critical genes have not been ruled out. Of further interest, patients with isolated congenital conotruncal heart defects and not diagnosed as DGS, VCFS, or CTAF were also shown to have microdeletions of this region in approximately 33% of the cases studied (Goldmuntz et al. 1993).

It has been suggested that the genes deleted in the region may be intimately involved in the interaction of neural crest derived cells with the endoderm of the pharyngeal pouches from which the affected structures are derived (Sharkey et al. 1992; Halford et al. 1993c). Such genes may play a key regulatory role in development, and may be sensitive to dosage effects, such as haploinsufficiency. Furthermore, Sharkey et al. (1992) proposed on the basis of the potential role of genes in the region that CES may be an overlapping syndrome with DGS. The hypothesis is that the phenotype demonstrated for CES is the result of increased dosage (3-4 copies) of the genes deleted in DGS. CES is associated with ear anomalies, heart defects and facial dysmorphism; these organs are derived from similar cell lineages and tissues as those affected by the haploinsufficiency syndromes of 22q11.2.

Several genes have been identified in the DGS region including ZNF74, a zinc-finger gene which encodes a putative transcriptional regulator (Halford et al. 1993c). This gene was found to be deleted in nearly all (95%) DGS patients studied. Another gene, TUPLE 1, has considerable homology with the yeast transcriptional regulator Tup1, and is another candidate for disease etiology (Halford et al. 1993b). Despite the concentrated efforts for identification of the major genes within the DGSCR, the potential role of flanking genes in the modification of the phenotypes of individuals

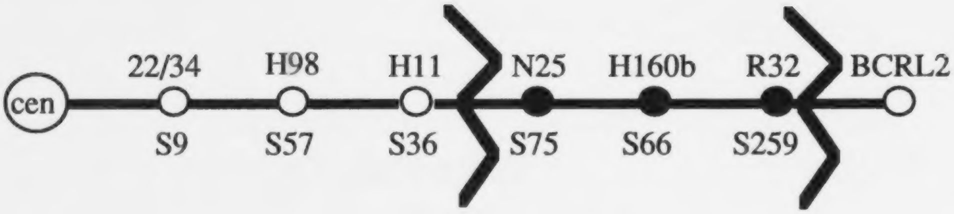

cen
22/34
H98
H11
N25
H160b
R32
BCRL2
S9
S57
S36
S75
S66
S259

Figure 4
The DiGeorge syndrome critical region (redrawn from Goldmuntz et al. 1993). Seven probes of 22q11.2 (represented by the circles) are shown proximal to distal from left to right. The probe names are shown above, and the locus numbers are shown below (without the D22 designation). The region between the two jagged lines represents the most frequently observed deletions for DGS and VCFS patients. Probes which are marked by the black circles are in the VCFS/DGS critical region.

with larger deletions cannot be ignored (Scambler 1993). The cause of these deletions is of interest. One study reported the presence of low copy repeats in and around the DGS region, and these were hypothesized to play a role in the genesis of deletions (and possibly duplications) in the region, akin to that observed in Charcot-Marie-Tooth type 1A (see below, Pentao et al. 1992).

These deletion syndromes of 22q11.2 are referred to as contiguous gene syndromes (Schmickel 1986) wherein several genes that are affected by the chromosomal defect (deletion or duplication) contribute to the overall phenotype of the syndrome. Like many of the contiguous gene syndromes , there is a considerable degree of phenotypic variability for DGS/CTAF/VCFS, even within families. Genetic background and stochastic influences may well prove to have a considerable effect on the phenotypic expression of these diseases.

Other syndromes associated with duplications

Down Syndrome

Down Syndrome (DS), typically associated with full trisomy 21, is one of the major identified causes of congenital heart disease and mental retardation (Epstein 1986). Other major features of DS include characteristic facies, skeletal anomalies, immune system anomalies and an increased risk of leukemia and Alzheimer-like presenile dementia. There is considerable phenotypic variability associated with DS, with only neonatal hypotonia and mental retardation occurring in 100% of trisomy 21 cases.

DS represents a meeting point for two opposing philosophies on the nature of aneuploidies and their phenotypes. Of the identified syndromes associated with aneuploidy, phenotypic variability within a specific syndrome and phenotypic overlap between different syndromes have been well documented. Shapiro (1983) argued that chromosomal imbalance leads to a general disturbance of the developmental processes, and this in turn results in a similar spectrum of defects that are observed with multiple distinct aneuploid conditions. In effect, this model proposed that phenotypic mapping of specific anomalies to specific regions of the genome would fail.

The reductionist approach, spearheaded by Epstein (1986, 1992), argued that imbalances of particular chromosomes or chromosomal regions lead to defined patterns of phenotypic characteristics. The phenotypic variability was argued to result from stochastic, environmental and genetic factors that may modify but do not obscure the overall pattern of phenotypic features. As to the occurrence of phenotypic overlap between different aneuploid states, it was proposed that due to the complexity of the developmental pathways, many gene products of different chromosomal origin are involved in any given pathway. Thus, it is of no surprise that mental retardation and growth impairment are almost universally associated with aneuploidies. Opitz (1982) stated that "human organs are evidently capable of responding to a high number of diverse dysmorphogenetic causes with the production of only a limited repertoire of malformations." In essence, phenotypic overlap is merely a reflection of the multifactorial developmental etiology of these systems. Under the principles of the reductionist approach, it is assumed that phenotypic mapping of specific anomalies to specific genomic locations is possible. Several such maps have been constructed for the Down syndrome region of chromosome 21.

Under the principles of phenotypic mapping as outlined by Epstein (1986, 1990), a given phenotypic anomaly may be assigned to a given region on the basis of its presence in concordance with the imbalance of this specific region, and the absence of the anomaly whenever the region was not in a state of imbalance. Due to phenotypic variability, the lack of a given phenotypic anomaly may be due to other factors rather than the lack of a direct gene effect, however, the presence of the phenotypic anomaly at a frequency comparable to that observed in typical larger imbalances is sufficient proof of direct effect (positive correlation of genotype to phenotype), and this is aided by the analysis of multiple individuals. It is likely that the strong, determinative loci are more readily mapped, as opposed to weak modifier genes that may slightly alter the frequency or form of the anomaly. Phenotypic mapping is tantamount to determination of the critical regions for individual characteristics of aneuploidy syndromes. Basically, the smallest region of overlap is defined with which all or many of the phenotypic characteristics are associated. It is very important to note that genes outside this critical region, when imbalanced, may still play a role in the etiology of the anomalous phenotypic traits. Once the critical region is defined potential candidate genes that contribute to the phenotype can be sought. Epstein (1986, 1990) suggests that the types of genes that may be more susceptible to gene dosage effects have products involved in regulatory pathways, multi-subunit molecules, intercellular interaction molecules, receptors, morphogens and growth factors. With all these principles in mind, the mapping of the Down Syndrome critical region has progressed considerably.

By the use of more unusual cases of DS associated with translocations and interstitial duplications (partial trisomy 21), the task of phenotype mapping and determination of critical regions is being pursued (Rahmani et al. 1990; Williams et al.

1990; Petersen et al. 1990). Using the principles of phenotype mapping previously mentioned, many of the classic DS features have been mapped to 21q22.1-qter. Figure 5 represents a phenotypic map for DS (Korenberg et al. 1992). Despite the preliminary delineation of the DS critical region, some features have been found to also map proximally on 21q. Mental retardation seems to be associated with several regions on 21q, but this is not particularly surprising.

The DS critical region has been further mapped by molecular methods to 21q22.3 in the vicinity of the D21S55 locus. The task of transcriptional mapping of the region has been initiated. Some 54 cDNA's have been isolated by Peterson et al (1994), spanning approximately 1 Mb of the critical region. The nature of these genes is yet to be determined.

DS and the principles of phenotype mapping used represent the model system by which phenotype mapping may be applied to the aneuploidy syndrome of CES. Unlike many of the aneuploidy syndromes, CES is complicated by the occurrence of both partial trisomy and tetrasomy of 22q11.2 which may modify the phenotypic picture. However, in contrast to DS, the phenotypic anomalies of CES are associated with a smaller duplication, which suggests that the effects are caused by one or a few genes.

Charcot-Marie-Tooth disease type 1A

Like CES, Charcot-Marie-Tooth disease, type 1A (CMT1A) is a syndrome typically associated with a small duplication. Furthermore, analysis of the duplication and a key gene within the critical region provides further insight on the possible mechanisms of duplication, mapping of disease, and the nature of gene dosage sensitivity.

First described in 1886, CMT1A is the most common form of peripheral neuropathy with a prevalence of 1 in 2500 (Skre 1974). CMT1A is clinically variable but may be characterized by distal muscle atrophy and weakness. At the cellular level, there is hypomyelination and Schwann cell proliferation which results in the most consistent diagnostic feature, the decrease in peripheral nerve conduction velocities (NCVs). Genetically, CMT1A is an autosomal dominant disease with age-dependent penetrance of most clinical features except the reduced NCVs which are age-independent. Vance et al. (1989,1991) demonstrated linkage of this disease to 17p11-12, and Lupski et al. (1991) determined that a submicroscopic duplication is typically associated with this disease. Several models of disease etiology such as dosage, gene

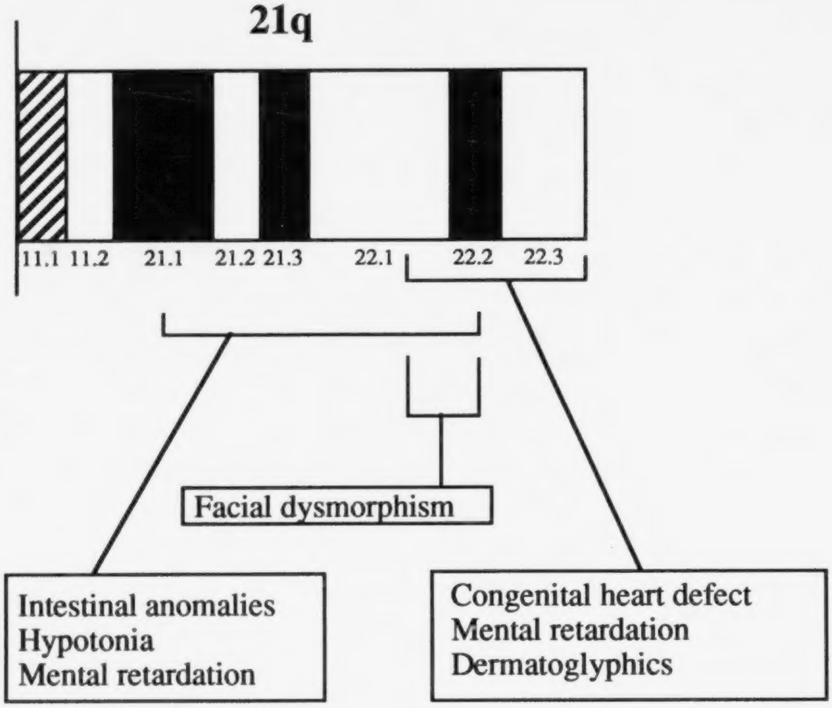

21q
11.1
11.2
21.1
21.2
21.3
22.1
22.2
22.3
Facial dysmorphism
Intestinal anomalies
Hypotonia
Mental retardation
Congenital heart defect
Mental retardation
Dermatoglyphics

Figure 5
The phenotypic map of Down syndrome (adapted from Korenberg et al. 1992). Figure shows an ideogram of the long arm of chromosome 21.The bars beneath the chromosome demonstrate the boundaries of the critical regions to which the specific phenotypic characteristics have been mapped. Note that mental retardation has been mapped to multiple regions of 21q.

disruption and position effect were considered, but gene dosage was implicated in CMT1A (Lupski et al. 1992). The duplication spans 3.1 Mb in the form of a directly repeated 1.5 Mb monomer. Large low-copy repeats (17-29 kb) flank the monomers and are presumably responsible for the formation of the duplication by unequal cross-over (Pentao et al. 1992). The level of recombination in the region is inordinately high (9 cM for 1.5 Mb).

The major gene of disease etiology that maps within the CMT1A duplication (Patel et al. 1992; Matsunami et al. 1992) is peripheral myelin protein 22 (PMP-22). The gene was recognized via previous identification of the mouse homolog (Suter et al. 1992a, 1992b) which was responsible for a CMT1A-like disease in rodents. The gene product is a 22 kDa integral transmembrane protein of the myelin sheath. Subsequent to the identification of the common CMT1A duplication (Wise et al. 1993), a wide spectrum of PMP-22 mutations have been identified associated with disease. However, an increase in dosage is sufficient to cause CMT1A. The reciprocal 1.5 Mb deletion results in hereditary neuropathy with liability to pressure palsies (HNPP), a milder distinct form of neuropathy(Chance et al. 1993). Point mutations resulting in dominant disease alleles (Valentjin et al. 1992) and recessive disease alleles (Roa et al. 1993) have been found.

The gene PMP-22 is sensitive to both increases and decreases in dose, resulting in diseases of distinct types. The actual role of PMP-22 protein in myelination has not been determined, although it appears to be play a key interactive role with other myelin proteins in the formation of the sheath. This protein represents one of the types of molecule that Epstein hypothesized would be dosage sensitive. As such, this gene, which represents probably one of twenty or so genes duplicated in the region, is the major contributory factor to this neuropathic disease.

The formation of the duplications and deletions via repeats represents an attractive model for the 22q11.2 deletion syndromes and may be a means by which the CES marker chromosomes are formed. Indeed, low copy specific repeats have been identified on chromosomes 15 and 22 (Donlon et al. 1986; Halford et al. 1993a), both of which can form marker chromosomes comprising of inverted duplications (Schreck et al. 1977; Schinzel et al. 1981a). The wide-spectrum of mutations of PMP-22 resulting in distinct clinical diseases raises interesting questions as to the role of genes on 22q11.2. Specifically, are the phenotypic manifestations of CES a clinically distinct but genetically related form of those of the deletion syndromes of 22q11.2?

Research Objectives

The primary objective of this research was to determine the size and structure of different duplications of 22q11.2 and to analyze their relationship to the CES phenotype. To characterize the duplications, a multi-method approach of dosage analysis was performed with numerous probes to loci mapped to 22q11.2. In this study, four 4 different groups of patients were analyzed, as outlined below. From these four groups, greater information could be obtained for the definition of the critical region (phenotype mapping), and for the characterization of the structure of the duplications. The basic questions that may be answered by analysis of the four distinct groups are outlined below.

(1) *Analysis of CES patients with dicentric marker chromosomes*

These cases represent the typical CES individuals with the appropriate phenotype and the presence of the supernumerary bisatellited marker chromosome. The key questions associated with the molecular analysis of these duplications were:

(a) are the duplications and associated breakpoints identical ?
(b) are the marker chromosomes symmetrical or asymmetrical ?
(c) by analysis of the distal boundaries of the duplications, what is the smallest region of overlap (SRO) of the duplications that is associated with the CES phenotype ?
(d) is the SRO or critical region of CES (CESCR) distinct from the critical region of DiGeorge syndrome ?
(e) in cases for which parental origin of de novo marker chromosomes can be determined, is it maternal or paternal origin ?

(2) *Individuals who are partially trisomic for 22q11.2*

These cases involve either cytogenetically detectable interstitial duplications or duplications derived from unbalanced products of a translocation. As they only demonstrate partial trisomy and not tetrasomy of 22q11.2, questions may be addressed on phenotype-dosage relationships.

(a) do the duplications involve the previously defined CESCR, and if so what is the relationship (if any) between dosage of genes in the region and severity of the phenotype ?

(b) are there common breakpoints involved in the formation of these duplications and do they localize to the regions identified in study (1) above ?

(c) is the proximal boundary of the CESCR further delineated by the proximal breakpoints associated with the interstitial duplications ?

(d) can parental origin be determined for these duplications and the type of exchange involved ?

(3) *Individuals with supernumerary ring chromosomes derived from chromosome 22*

These cases represent more unusual forms of duplications, and as such are of particular interest in terms of their structure.

(a) are the breakpoints associated with ring formation similar to those identified for other duplications ?

(b) is the size of the duplication smaller than previously identified, and if so does this duplication further delineate the distal boundary of the CESCR ?

(c) what is the relationship between dosage and phenotype in these cases ?

(4) *Individuals with apparent CES but normal karyotypes*

After identification of the CESCR with the three studies above, these individuals were then studied with probes to loci within the CESCR, to establish whether there is evidence of any small interstitial duplications of the CESCR to explain the presence of the CES phenotype?

Materials and Methods

Clinical and Cytogenetic Evaluation

Clinical evaluations of the patients were performed by the referring physicians, the appropriate specialists, or both. The clinical information was obtained from the literature for the published cases; CM01 (Rosenfeld et al. 1984), CM05 and CM06 (cases 2 and 5, respectively, in Schinzel et al. 1981), CM10 (Buckton et al. 1985), CE01 (Reiss et al. 1985), CE02 (Knoll et al. 1994), and CE03 (Bröndum-Nielsen 1991).

Cytogenetic analysis was performed using standard techniques at the respective clinical cytogenetics laboratories.

Human Cell Culture

Incubation Conditions:

All human cell cultures were grown in a 37°C, 5% CO_2 incubator. A list of the cell samples from individuals studied is given in Table 5.

Suspension cultures:

Established lymphoblastoid cell lines were cultured in RPMI 1640 media (Gibco/BRL) supplemented with 10% fetal bovine serum (Gibco/BRL), 1% L-glutamine (200 mM stock, Gibco/BRL) and 1% penicillin/streptomycin (stock 5000 U/ml, Gibco/BRL). The passage of such cells was achieved by dilution, typically at 1:4 or 1:5. Suspension cultures were grown in T25 flasks (Corning), in a volume of 5-10 ml/ flask.

Monolayer cultures:

Fibroblasts were cultured in DMEM media (Gibco/BRL) supplemented with 10% fetal bovine serum, 1% L-glutamine and 1% penicillin/streptomycin. Chorionic villus cells were cultured in MEM media (Gibco/BRL) supplemented with 20% fetal bovine serum, 1% L-glutamine and 1% penicillin/streptomycin. Amniocytes were cultured in 20% Chang media (α-MEM from Gibco/BRL, Chang supplements A and B from Irvine Scientific) supplemented with 1% L-glutamine and 1% penicillin/streptomycin. Monolayer cultures were grown in T25 and T75 flasks

Table 5 Cell sample summary

A complete listing of all the samples from individuals that were used in this study. The samples are divided into six categories (a-f) based on their use in the studies. The numerical coding system used is for ease of identification (as opposed to the original laboratory identification of individuals). The parents coding is based on the coding given to the affected offspring. For example, the parents of CM02 are given the code YM02 (Y=father, M02=offspring code), and XM02 (X=mother, M02=offspring code). The different cell types referred to in the table are as follows:- Lymph = lymphoblastoid cell line, Fibro = fibroblasts, CVS = chorionic villus sample, Blood = only available resource was a whole blood sample, Amnio = amniocytes. "Original source" refers to the individual (typically the referring clinician) who either collected and/or made the original samples available. The "cell line source" refers to the individual who either established a lymphoblastoid cell line or who established the growth of and provided the skin, chorionic villus, or amniocyte samples for analysis. NIGMS refers to the Human Genetic Mutant Cell Repository at the Coriell Institute of Medical Research, Camden, MD.

(a) Controls

Lab ID	Cell type	Notes	Original Source	Cell line source
MT(NC1)	Lymph	Normal	Dr.S.Shapira	Dr.H.E.McDermid
GM03657	Fibro	Normal	-	NIGMS
GM07106	CVS	Trisomy 22	-	NIGMS
GM02325	Fibro	Partial trisomy 22	-	NIGMS

(b) Individuals with marker chromosomes

Lab ID	Code	Cell type	Original Source	Cell line source
BZ	CM01	Lymph	Dr.R.S.Verma	Dr.A.M.V.Duncan
JD	CM02	Lymph	Dr.C.R.Greenberg	Dr.H.E.McDermid
KS	CM03	Lymph	Dr.W.J Rhead	Dr.J.Biegel
MM	CM04	Lymph	Dr.R.Stallard	Dr.A.M.V.Duncan
S2	CM05	Lymph	Dr.A.Schinzel	Dr.A.M.V.Duncan
S5	CM06	Lymph	Dr.A.Schinzel	Dr.A.M.V.Duncan
GM	CM07	Lymph	Dr.J.Siegel-Bartelt	Dr.J.Siegel-Bartelt
JM	CM08	Lymph	Dr.J.Siegel-Bartelt	Dr.J.Siegel-Bartelt
IG	CM09	Lymph	Dr.M.Baraitser	Dr.A.M.V.Duncan
ISCA	CM10	Lymph	Dr.V.Van Heyningen	Dr.V.Van Heyningen
MG	CM11	Lymph	Dr.B.S.Emanuel	Dr.B.S.Emanuel
CO	CM12	Blood	Dr.L.Jenkins	n/a

(c) Individuals with partial trisomy 22 from an interstitial duplication or a duplication resulting from a translocation

Lab ID	Code	Cell type	Original source	Cell line source
LW	CE01	Lymph	Dr.R.E.Magenis	Dr.H.E.McDermid
SK	CE02	Lymph	Dr.S.Thallur	Dr.H.E.McDermid
BE	CE03	Lymph	Dr.M.Nordenskjöld	Dr.M.Nordenskjöld
BF	CE04	Amnio	Dr.J.Chernos	Dr.J.Chernos

(d) Individuals with apparent CES but a normal karyotype

Lab ID	Code	Cell type	Original source	Cell line source
AL	CN01	Lymph	Dr.A.McConkie-Rosell	Dr.J.Biegel
JW	CN02	Lymph	Dr.J.Siegel-Bartelt	Dr.J.Siegel-Bartelt
WH	CN03	Blood	Dr.P.Ferreira	n/a

(e) Individuals with supernumerary ring (22) chromosomes

Lab ID	Code	Cell type	Original source	Cell line source
25181	CM13	Lymph	Dr.S.R.Patil	Dr.S.R.Patil
25117	CM14	Lymph	Dr.S.R.Patil	Dr.S.R.Patil
25105	CM15	Lymph	Dr.S.R.Patil	Dr.S.R.Patil
ME	CM16	Lymph	Dr.Y.Fukushima	Dr.Y.Fukushima

(f) Clinically normal parents

Lab ID	Code	Cell type	Original source	Cell line source
RD	YM02	Lymph	Dr.C.R.Greenberg	Dr.H.E.McDermid
ED	XM02	Lymph	Dr.C.R.Greenberg	Dr.H.E.McDermid
JS	YM03	Lymph	Dr.W.J.Rhead	Dr.J.Biegel
LS	XM03	Lymph	Dr.W.J.Rhead	Dr.J.Biegel
YMG	YM11	Lymph	Dr.C.Schultheis	Dr.J.Biegel
JK	YE02	Blood	Dr.S.Thallur	n/a
NK	XE02	Blood	Dr.S.Thallur	n/a

(Corning) and 150 mm tissue culture dishes (Falcon). For each passage (1:3 to 1:5) of monolayer cultures, cells were washed with Hank's Balanced Salt Solution (Gibco/BRL) to remove serum-based trypsin inhibitors. The cells were then treated with a minimal volume of trypsin-EDTA (Gibco/BRL) to detach them from the surface. After a typical treatment of 1-2 minutes, the detached cells were appropriately diluted in medium and aliquoted.

Freezing of cell cultures:

Cell samples from the early passages of cell lines were frozen in liquid nitrogen. Typically one T25 flask of a suspension culture or one T75 of trypsinized monolayer cells were pelleted at 300 x g for 10 minutes. The pellets were then resuspended in 1 ml of freezing medium (fetal bovine serum with 5% dimethyl sulfoxide from Sigma) and aliquotted into a cryotube (1.5 ml capacity from Nunc). The resulting tube was placed over liquid nitrogen for approximately one hour then transferred to a liquid nitrogen tank. Such aliquots were thawed and resuspended in appropriate media when required.

EBV Transformation

Epstein-Barr Virus (EBV) transformation of B-cells was used to establish permanent lymphoblastoid cell lines for analysis. The following lab protocol was used:

(1) *Isolation of lymphocytes from blood*

Approximately 5 ml of blood was transferred to a 15 ml sterile tube (Corning) and was spun at 1000 x g in a Jouan centrifuge (Canberra Packard) for 5 minutes. The "buffy coat" of lymphocytes and some serum above it was then removed to a fresh 15 ml tube and made up to 4 ml in volume with RPMI 1640 medium (Gibco/BRL). This sample was then layered over 4 ml of Ficoll-paque (Pharmacia) in a 15 ml tube. Separation of lymphocytes in the Ficoll-paque was achieved by spinning the sample at 600 x g (slow acceleration) for 20 minutes. The separated lymphocytes were then washed three times with RPMI 1640 medium (pelleted at 200 x g for 8 minutes).

(2) *Transformation*

The lymphocyte pellet was resuspended in 5 ml of RPMI 1640 medium supplemented with 1% L-glutamine, 1% penicillin/streptomycin and 16% fetal bovine serum and incubated for 4-8 hours. Then $6x10^5$ transforming units of EBV (Showa University Research Institute for Biomedicine, CA) were added, as well as cyclosporin (final concentration of 0.2 mg/ml). The culture was left for 3-4 weeks and observed for changes in medium colour and formation of large clumps (indications of growth). When successful, the established cell line was expanded, subcultured and used for analysis . Early passages of the cells were frozen in liquid nitrogen.

T-cell expansion using Interleukin-2

This technique was employed to expand rapidly a culture of T-cells isolated from whole blood as an alternative to EBV transformation, which was not as reliable. The procedure described in detail by Adolph et al. (1988) routinely expanded the number of T-cells by 20-fold or greater in only two weeks using the lymphokine Interleukin-2 (Gibco/BRL). This procedure enabled successful preparation of genomic DNA and metaphase spreads from small blood samples. The slightly modified protocol used is described below.

(1) *Isolation of lymphocytes*

About 2-3 ml of whole blood (less than 3 days old) was layered over 2 ml of Ficoll-paque (Pharmacia) in a 15 ml tube (Corning) and spun at 600 x g for 15 minutes in a Jouan centrifuge (Canberra Packard). The separated layer of lymphocytes ("buffy coat") was transferred to a clean tube and washed twice in RPMI 1640 medium, being pelleted at 200 x g for 8 minutes each time.

(2) *Culturing in PHA Medium*

The lymphocytes were resuspended and incubated for 4 days in 5 ml of RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% penicillin/streptomycin, 1% sodium pyruvate and 1% phytohemagglutinin M (Gibco).

(3) *Addition of interleukin-2*

On the fourth day, the cells were dispersed and counted with a hemocytometer. The cells were then subcultured to a density of $2x10^5$ cells/ml (usually a final volume of 10 ml) and interleukin-2 was added to a final concentration of 10 units/ml.

(4) *Expansion of culture*

The cells were subcultured and maintained at a density of approximately $2x10^6$ cells/ml in the PHA medium supplemented with interleukin-2. After 12-14 days the expanded T-cell culture was harvested and genomic DNA extracted with an average yield in the 100-200 μg range. Alternatively, after 6-8 days, the T-cell culture was used for preparation of metaphase spreads (see fluorescence in situ hybridization - preparation of slides).

Human Genomic DNA Preparation

DNA was extracted from human cells by two different procedures. The first method involved overnight lysis followed by several extractions using organic chemicals, the second method utilized cationic detergents and high salt concentrations for rapid lysis and selective precipitation of DNA.

Method I: Lysis and Phenol/Chloroform Extraction

This protocol represents a modification of the procedure described in Sambrook et al. (1989).

(1) *Pelleting of cells*
Cells from 10-20 T25 flasks (lymphoblasts), 10-15 150 mm dishes (monolayer cultures) or the lymphocytes isolated from 20-50 ml of blood were pelleted by centrifugation (300 x g for 10 minutes).

(2) *Washing cells*
Cells were washed once or twice with Hanks Balanced Salt Solution.

(3) *Lysis of cells*
Cells were resuspended in 4.5 ml of 10/10/10 (10 mM Tris pH 7.5/ 10 mM NaCl/ 10 mM EDTA). While swirling the suspension, 500 µl of 10% SDS was added to lyse the cells. Finally, 100 µl of proteinase K (10 mg/ml stock, Boehringer Mannheim Canada) was added. The mixture was incubated for 16-24 hours at 37°C.

(4) *Extraction of DNA*
The lysed cell suspension was extracted two or three times with an equal volume of phenol/chloroform. Mixing was by gentle inversion. The aqueous suspension containing the DNA was then transferred to a clean tube.

(5) *Precipitation of DNA*

A 1/10th volume of 3 M sodium acetate (pH 4.5) was added, followed by 2-2.5 volumes of -20°C 95% ethanol. The mixture was rocked back and forth until the DNA precipitated as a white sticky wad. This DNA was removed by a plastic pipette tip and transferred to a 1.5 ml microfuge tube. DNA was washed with 70% ethanol, then briefly dried under vacuum.

(6) *Resuspending DNA*

The dried pellet was resuspended in 0.5-1.0 ml of TE (10 mM Tris pH 8.0/ 1 mM EDTA) to a final concentration of 0.5-2.0 μg/μl. The average yield of DNA was 0.75-1.50 mg for lymphoblasts, 0.50-1.00 mg for fibroblasts, and 0.20-0.30 mg for blood.

Method II: Cationic Detergent/High Salt Extraction

This procedure was modified from the original procedure described by Gustincich et al. (1991) for extraction of DNA from 0.3 ml of whole blood. The modifications enabled scaled-up extractions of genomic DNA.

(1) *Preparation of cells*

Either 5 ml of blood (collected in sodium heparin tubes) or pelleted cells from 4-6 T25 flasks (lymphoblast) or 8-10 T75 flasks (monolayer cultures) were required. Cells from established cultures were resuspended in 5 ml of Hanks Balanced Salt Solution. The samples were transferred to 50 ml tubes (Corning).

(2) *Lysis of cells*

To lyse the cells, 10 ml of lysis buffer was added to the whole blood or resuspended cultured cells. The lysis buffer comprised of 8% DTAB (Sigma) in 1.5 M NaCl, 100 mM Tris-HCl pH 8.6, 50 mM EDTA. The mixture was then incubated at 68°C for 10-15 minutes. At this stage, the sample was sometimes frozen for later use. In these cases, the heating step was repeated.

(3) *DNA extraction*

Immediately upon adding 15 ml of chloroform to the hot solution, the tube was inverted 5 or 6 times before the mixture was transferred to 50 ml Oakridge tubes (Nalgene) for spinning at 15000 x g in a HB4 Sorvall Rotor for 15 minutes.

(4) *DNA Precipitation I*

The upper aqueous phase was transferred to a clean Oakridge tube and 15 ml of sterile deionized distilled water added for a total volume of approximately 30 ml. [Note:- If the sample was difficult to aspirate without contamination from the white interface, the extraction step (3) was repeated]. Next approximately 1.7 ml of CTAB precipitation buffer was added (to a final concentration of 0.3% CTAB). The precipitation buffer comprised 5% CTAB (Sigma) in 0.4 M NaCl. The mixture was inverted twice, then the DNA/CTAB complex was pelleted at 15000 x g for 10 minutes in the HB4 rotor of a Sorvall centrifuge.

(5) *DNA/CTAB Resuspension*

The DNA/CTAB pellet was resuspended in 5 ml of 1.2 M NaCl for 3-4 hours by gently mixing at 37°C to remove the detergent from the DNA.

(6) *DNA Precipitation II*

To the suspension, 18 ml of ice cold 95% ethanol was added, the mixture was inverted and the precipitated DNA removed to a fresh 1.5 ml tube (or briefly pelleted if necessary).

(7) *DNA Resuspension*

The pellet was briefly dried under vacuum then resuspended in 300-500 ml of TE pH 8.0 with a final concentration of 0.5-1.0 μg/μl. The average yield for this procedure was 200-400 μg.

The DTAB/CTAB method was a more rapid and efficient method of genomic DNA extraction. Furthermore, the use of these cationic detergents enabled safer extraction from whole blood samples due to the reduction of handling steps (no lymphocyte isolation) and less use of organic extractions. The procedure resulted in less sheared DNA samples with very low concentrations of RNA.

Preparations of plasmid, cosmid, and bacteriophage DNA

Growth medium for bacterial cultures:

Escherichia *coli* cultures were grown in Luria-Bertani medium (10 g Bacto-tryptone/5 g yeast extract/10 g NaCl per liter) for cosmid/plasmid DNA preparation with the appropriate concentration of antiobiotic. For bacteriophage λ 2001 DNA preparations, the E. *coli* cultures were grown in NZCYM medium (10 g NZ Amine/5 g NaCl/5 g Bacto-yeast extract/1 g casamino acids/2 g $MgSO_4.7H_2O$ per liter).

Plasmid and cosmid DNA preparation:

Two commercially available kits were used. (a) The Wizard (Magic) MaxiPreps DNA Purification System (Promega) and (b) Qiagen-tip 500 (Qiagen Inc). The provided protocols were followed for each of these kits and as such are not described in further detail. Both systems gave an average yield of 500 µg of plasmid DNA from an E. *coli* culture of 100-250 ml. An average yield of 200 µg of cosmid DNA was achieved from a 500 ml E. *coli* culture with the Qiagen-tip 500. The cosmids were all isolated from the chromosome 22-specific cosmid library LL22NC03 (Lawrence Livermore National Laboratory, Human Genome Center, Livermore, CA). This library comprises approximately 12,000 individually picked cosmid clones which are gridded into 130 96-well dishes.

Large-scale bacteriophage DNA preparation:

The procedure used for the preparation of bacteriophage λ DNA from E. *coli* lysates is described in detail in Sambrook et al. (1989).

Small-scale preparations of plasmid DNA:

The method described by Serghini, Ritzenthaler and Pinck (1989) was used to isolate 3-5 µg of plasmid DNA from 1.5 ml E. *coli* cultures.

Restriction digests, electrophoresis and Southern blotting

DNA digestion with restriction endonucleases:

Genomic DNAs were digested with the appropriate restriction endonucleases under conditions recommended by the manufacturers (BRL, Pharmacia and New England Biolabs).

Agarose gel electrophoresis:

Digested DNAs were separated by agarose gel electrophoresis in 1 X TBE (0.089 M Tris / 0.089 M Boric acid / 0.002 M EDTA / pH to 8.4). Percentage of agarose and running time varied according to the separation required for the digested DNAs. Typically, gels were 0.8% agarose (containing 0.5 µg/ml of ethidium bromide) and were run at 1-1.5 V cm^{-1} for 16-20 hours.

Southern Blotting:

Transfer of DNA from agarose gels to Genescreen Plus membrane (DuPont) was achieved using the capillary method described by Southern (1975). The DNA was acid-nicked by treatment with 0.25 M HCl for 10 minutes, then rinsed in water. The gels were then soaked with gentle agitation in denaturing solution (1.5 M NaCl / 0.5 M NaOH) 2 x 30 minutes. The gel was then rinsed in water and soaked in neutralizing solution (1.5 M Tris / 1.5 M NaCl / pH 7.5) for 45 minutes. The gel was then flipped (wells down) and placed on a prewetted (10 X SSC) wick of three strips of Whatmann 3MM filter paper wrapped around a raised glass plate. The following were layered on the gel in order: Genescreen Plus membrane (prewetted in 10 X SSC), 3 pieces of dry Whatmann 3MM paper (size of gel), 10 pieces of blotting paper (GB003 from Schleicher and Schuel), 30-40 paper towels, a glass plate and a weight (approximately 250 g). The glass reservoir was filled with 10 X SSC (1.5 M NaCl/0.15 M Sodium Citrate), and the whole apparatus wrapped in Saran wrap to avoid evaporation. After 16-20 hours of transfer the membrane was washed in 0.4 M NaOH for 60 seconds, followed by a 5 minute neutralization in 0.2 M Tris/2 X SSC. The membrane was then air-dried at room temperature prior to use.

DNA probes, hybridization and autoradiography

DNA probes:

A summary of all the DNA probes used in this study is provided in Table 6. The relative order of the 22q11.2 probes is given in Figure 6, and was derived from physical mapping of the loci in McDermid et al. (submitted) and genetic mapping by Fibison et al. (1990). The 38F3 cosmid is included for the completeness of the physical map only (Figure 6). Failure to isolate unique sequences that were not prone to change in copy number in all individuals (including controls) precluded its use in DNA dosage analysis. This phenomenon was seen in no other locus and may be related to the proximity of 38F3 to the centromere (Xie et al. 1994).

Radioactive labelling of probes:

DNA probes were isolated by digestion with the appropriate enzyme and purified by gel electrophoresis in 0.8% low melting point agarose (Sea Plaque, FMC). These probes were then directly labelled with $\alpha[^{32}P]$ dCTP (ICN) by the random primer method (Feinberg and Vogelstein 1984). Each labelling reaction comprised 25 µl (30-100 ng) of denatured probe DNA in undiluted agarose after 10 minutes of boiling; 2 µl

Table 6 Information summary on DNA probes

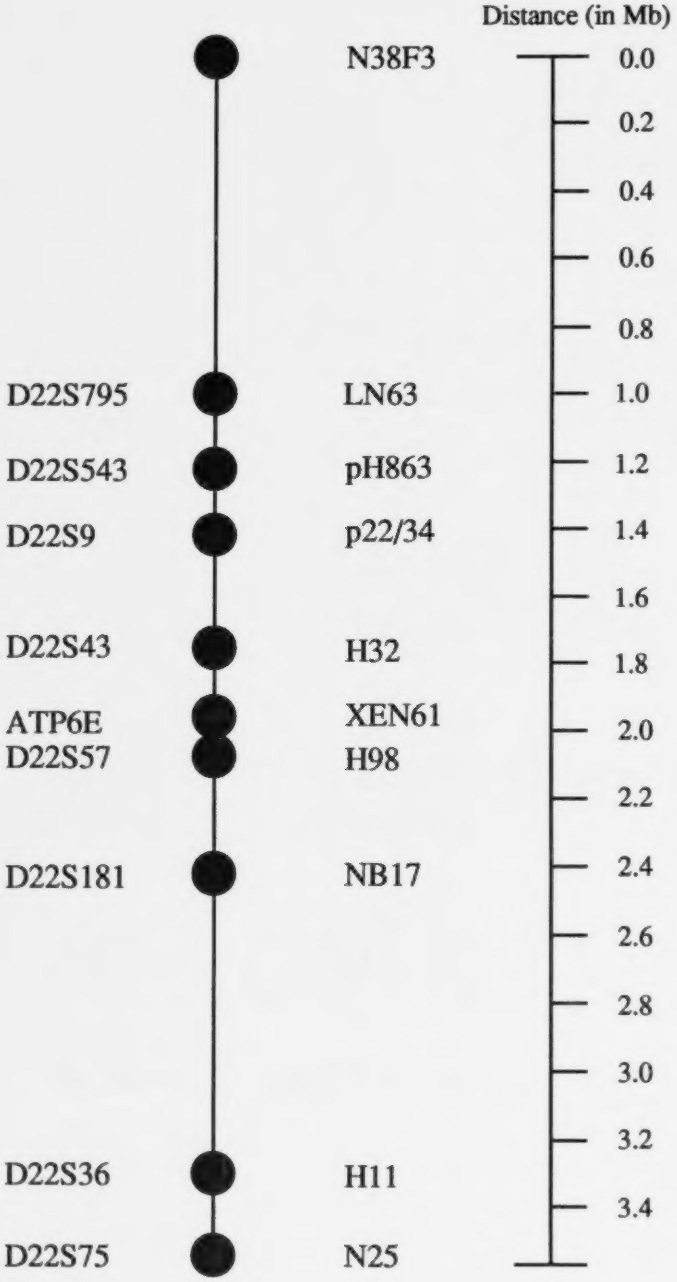
Locus	Probe	Insert	Cosmid	Polymorphism	Reference
D22S9	p22/34	R/H 1.8	107D6	TaqI A1(5.8),A2(3.2)	McDermid et al. (1986)
D22S36	H11	H 1.0	103A2	MspI A1(3.3),A2(1.6) constants (3.7,2.3)	Budarf et al. (1991)
D22S37	H13	H 1.6	-	-	Budarf et al. (1991)
D22S39	H17	H 3.5	108A7	-	Budarf et al. (1991)
D22S43	H32	H 1.5	-	TaqI A1(4.8),A2(3.8), A3(2.9)	Budarf et al. (1991)
D22S44	H35	H 1.0	-	-	Budarf et al. (1991)
D22S57	H98	H 0.7	-	BstXI A1(2.6),A2(2.0) MspI B1(2.5),B2(1.5)	Budarf et al. (1991)
D22S75	N25	H 2.4	5D9	TaqI A1(3.3),A2(1.0) constant (1.6)	McDermid et al. (1989)
D22S181	NB17	R/H 0.7	54G12	TaqI A1(2.9),A2(2.2)	Lekanne-Deprez et al. (1991)
D22S318	DAC1	R 1.4	-	-	Lamour et al. (1993)
D22S543	pH863	(0.3)	-	-	Hudson et al. (1995)
D22S795	N63	(0.3)	-	-	Bell et al. (1995)
ATP6E	XEN61	X/N 1.0	4D9	-	Baud et al. (1994)
-	N38F3	-	38F3	-	Xie et al. (1994)
D21S15	pGSE8	T 2.0 R 6.3	-	-	Stewart et al. (1985a)
D21S19	pGSB3	M 1.1 R 6.4	-	-	Stewart et al. (1985b)
D21S110	p21-4U	H 3.0	-	-	Spinner et al. (1989)

Locus designation is given in the first column. The laboratory name for the probes to the specific loci is given in the second column. "Insert" refers to the size of the probe (in kb) when cut out of the vector with the appropriate restriction enzyme. R = EcoRI, H = HindIII, X = XhoI, T = TaqI, M = MspI, and () indicates that the probe is a PCR product. "Cosmid" refers to the address of cosmids isolated from the Lawrence Livermore chromosome 22, gridded cosmid library, LL22NC03 (Human Genome Center). Addresses were provided by either Dr.Marcia Budarf or Kerry McTaggart. Polymorphism data is provided only for the probes for which RFLP analysis was performed. The polymorphic enzyme, alleles produced , allele sizes (kb) and constant bands are given. The original source of the probe is given in the "reference" column.

Figure 6 Physical map of the 22q11.2 loci

The relative order of the 22q11.2 loci (denoted by black circles) is shown, proximal to distal (top to bottom). The locus identification is shown to the left, the probe names are shown to the right. The N38F3 cosmid represents the most proximal probe available. It was isolated after two cosmid walks away from the pericentromeric α-satellite sequences (Xie et al. 1994). The scale bar to the far right indicates the approximate distances involved. These distances are based on maximum estimates from physical mapping in the region (adapted from McDermid et al., submitted).

Figure 6 Physical map of the 22q11.2 loci


Distance (in Mb)
N38F3
D22S795
LN63
D22S543
pH863
D22S9
p22/34
D22S43
H32
ATP6E
XEN61
D22S57
H98
D22S181
NB17
D22S36
H11
D22S75
N25
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
2.4
2.6
2.8
3.0
3.2
3.4

of bovine serum albumin (10 mg/ml, high grade, New England Biolabs); 10 µl of oligo labelling buffer (100 µl 1 M Tris, 12.5µl 1 M $MgCl_2$, 2.5 µl 50 mM dATP, 2.5 µl 50 mM dGTP, 2.5 µl 50 mM dTTP, 250 µl 2 M Hepes pH 6.6, 150 µl 90 A_{260} units/ml random primers, and 2 µl of b-mercaptoethanol); 5-10 ml of $\alpha[^{32}P]$ dCTP (50-100 µCi); sterile distilled water (up to 48 µl); and 2 µl (20 units) of Klenow fragment (Pharmacia). The reaction mixture was incubated at 37°C for 2-3 hours and then stopped by adding 50 µl of stop buffer (50 mM EDTA/50% glycerol/blue dextran). It was then passed through a Sephadex G-50 column to remove unincorporated radioactive nucleotides. Prior to hybridization, single copy (unique) probes were boiled for 10 minutes with 100 µl of heterologous DNA (10 mg/ml sonicated herring sperm DNA) for every 10 ml of hybridization solution. Probes that contained repetitive sequence were preannealed to a vast excess of total human DNA prior to hybridization. This procedure was developed by Litt and White (1985) to compete out repetitive sequences. To the probe (in approximately 200 µl), 500 µl of sonicated human placental DNA (2.5 mg/ml, average size 500 bp) and 84 µl of 1 M sodium phosphate (pH 7.0) were added. The probe/competitor mixture was then boiled for 10 minutes followed by an incubation of 4-6 hours at 65°C to enable preferential annealing of repetitive sequences to take place. The probe was then ready for hybridization. If necessary, denatured radioactively labelled wild-type λ DNA was added to visualize size marker DNA.

Hybridization:

Hybridizations were performed at 65°C for 18-24 hours in a roller bottle hybridization oven (Tyler Research) with 6.6% SDS, 1 mM EDTA, 0.25 M sodium phosphate pH 6.5, 4.7 X Denhardts solution (slight modification of Church and Gilbert 1984). After hybridization, blots were washed with 2 X SSC/0.2% SDS at room temperature for 10 minutes, 0.2 X SSC/0.2% SDS at 65°C for 10 minutes, followed by an optional wash with 0.1 X SSC/0.2% SDS at 65°C for 5-10 minutes. Blots were then placed and sealed in non-static plastic pouches (Baxter).

Autoradiography:

Blots were exposed to Kodak XAR-5 film at -70°C for an appropriate length of time (typically 24-48 hours). The film was processed according to manufacturer's recommendations (Kodak).

Rehybridization:

Prior to rehybridization, bound probe was removed by boiling the blots for 20-25 minutes in a large volume of 0.1 X SSC/1% SDS (as recommended by DuPont).

Quantitative Analysis of Autoradiograms:

Quantification of probe signals from the autoradiograms was carried out by two systems: (1) Gelprint 2000i with Gelprint Toolbox software (Biophotonics), and (2) GS670 Scanning Densitometer with Molecular Analyst software (Biorad). Both systems were used to quantify autoradiographic bands by measuring the sum density (optical density x area), calculated as an average measurement through the entire bands (profile). This method was more accurate than a narrow line scan through the bands because the bands are subject to minor fluctuations of density across their breadth which can lead to different values depending on where the line is traced.

Dosage Analysis Methods

Two methods were utilized to determine copy number of 22q11 test probes - *quantitative dosage analysis* and *RFLP analysis.*

(I) Quantitative dosage analysis

Quantitative hybridization is associated with variables that makes copy number determination problematic (see Appendix 1). Due to this variability, using a low number of replicates was determined to be relatively unreliable. Therefore a large number of replicates was used to reduce errors and to enable statistical analysis to be performed. Four to seven replicates of DNAs from a normal disomic control, a chromosome 22q11.2 trisomic control and DNAs from two or three test individuals were loaded onto each gel. Concentrations of DNA samples were previously determined such that approximately equal amounts were loaded into each lane. The resulting Southern blots of these gels were hybridized to the test probe(s) and a non-syntenic reference probe. Hybridization signals were then quantified from the autoradiograms. Standardized signal ratios were calculated as the ratio of the test probe to the reference probe, thereby correcting for variable DNA concentration between lanes. Therefore, for each test probe, data sets of 4-7 standardized ratios were established for each of the disomic and trisomic control DNAs and for the respective CES patients. If nonspecific background interfered with the analysis of individual bands on an autoradiogram, those affected ratio values were eliminated from the data set.

Statistical Analysis:

The data sets analyzed in this study were statistically compared by the Wilcoxon Rank Sum test. Due to the variability of hybridization and small sample sizes (less than 10), this nonparametric test was considered the most appropriate. The test required no assumptions on the distribution within the data sets but only tested whether the two populations of ratios being tested are the same. In general, the control data set (disomic or trisomic) is grouped with the test data set. The grouped values are ranked from smallest to largest on the basis of their numerical value. The rank values assigned to the two data sets are then summated and compared. The more similar the data sets, the greater the degree of overlap and the closer the sum rank values are. Whether this difference is significant or not is determined by comparing these values to the appropriate statistical tables (for greater detail on the Wilcoxon Rank Sum test, see Appendix 2).

Copy number determination procedure:

(1) The disomic and trisomic control data sets were compared to determine if three copies could be distinguished from two copies in a given hybridization event. If the sum of the ranks of the trisomy data set (SR_T) was significantly greater (level of significance, $p=0.05$) than that of the disomic data set (SR_D), then the patient data could be analyzed. If the control sets did not significantly differ then all associated data was eliminated. This was a rare event caused by unusually discrepant values (see Appendix 1).

(2) For each of the test loci, an assumption was made that each patient possessed two, three or four copies of the sequence. The patient data sets (SR_P) for the test locus were compared to the data sets of the controls. Copy number was determined from the statistical analysis by using the following criteria:-

(i) two copies, if SR_P is not significantly greater than the SR_D, and is significantly less than the SR_T

(ii) three copies, if the SR_P is significantly greater than the SR_D, but is not significantly greater than the SR_T

(iii) four copies, if the SR_P is significantly greater than both the SR_D and SR_T

In all cases, a probability of 0.05 was used as the level of significance.

(II) RFLP Analysis

In RFLP analysis, three or four copies are more readily discernible, because in heterozygotes, the duplication is distributed between two or more alleles. This distribution results in a higher relative signal ratio than is obtained by method (I) above. For example, three copies may be represented by two alleles in a heterozygote with a 2:1 signal ratio or a 100% signal difference. If the same duplication is quantified using test and reference probes, a 3:2 signal ratio is observed with only a 50% signal difference. There is no need for a reference locus to standardize for DNA concentration with RFLP analysis, because the alleles of a given polymorphic locus act as internal controls. However, in the present study, a heterozygote that showed two alleles of equal intensity was inconclusive indicating either two or four copies of this locus. These cases could only be resolved by test / reference probe dosage analysis or by the additional hybridization of a control probe to the RFLP blot. The major drawback of RFLP analysis is the requirement for heterozygosity.

When tested, the sum density of the polymorphic bands were quantified from autoradiograms as previously described. In each case, three or four replicates were used and the ratios calculated between between the alleles of each heterozygote. The allelic ratio was compared to heterozygote controls (disomic/trisomic) and copy number determined using the Wilcoxon Rank Sum test.

RFLPs and determination of parental origins of duplications

In addition to dosage analysis of individuals, RFLPs enable identification of specific alleles. With parental samples available, it may be possible to identify the parental source of the duplicated alleles. Such a process is dependent on whether the alleles demonstrate an informative pattern for a given polymorphic locus.

Fluorescence in situ hybridization (FISH) with cosmids

Preparation of slides for FISH:

The following protocol was used to generate a high proportion of metaphase cells for the preparation of slides for fluorescence in situ hybridization analysis (FISH).

(1) *Synchronization of cells*

Lymphoblasts were synchronized by the use of methotrexate as described elsewhere (Dracopoli et al. 1994). Three T25 flasks of cells were fed with fresh medium and allowed to incubate at 37°C for a further 24 hours. Methotrexate was added to a final concentration of 0.45 μg/ml, which constituted the block stage. The cells were incubated at 37°C for approximately 17 hours (synchronization). The medium was then removed and fresh medium with excess thymidine was added (final concentration of 0.24 μg/ml). This constituted the block removal stage. The three flasks of cells were then incubated for either 4 hours and 15 minutes, 4 hours and 45 minutes or 5 hours and 15 minutes, with colcemid being added for the last 15 minutes of the incubation (0.05 μg/ml).

(2) *Preparation of cells for slides*

The cells were transferred to a 15 ml tube (Corning) and spun at 300 x g for 10 minutes. The cell pellet was resuspended in 10 ml of 75 mM KCl and incubated for 10 minutes at 37°C, causing the cells to swell and the membrane to weaken. The cells were then pelleted once again and supernatant discarded. The pellets were resuspended in a small volume of the salt solution and then 10 ml of a 3:1 methanol:glacial acetic acid fixative was added, the first 2 ml being added slowly due to the now delicate nature of the cells. The tube was gently inverted 2 or 3 times and then the cells re-pelleted. The fixing step was repeated twice more with the final pellet being resuspended in an appropriate volume of fix (1-2 ml). These preparations of fixed cells were then stored at -20°C

(3) *Preparation of slides*

Two to three drops of the fixed cells were dropped onto a clean, wet, cold glass slide (cleaned in 95% ethanol and stored in clean water at 4°C prior to use). Typically, to produce good metaphase spreads and to separate the chromosomes of these cells, the cell suspension was dropped from a height of approximately 24 inches, with the slides tilted at an angle of 45 degrees (to aid spread of solution and cells across the slide). The slides were then placed over a 70°C water bath for a few minutes. The slides were checked for quality and quantity of metaphase spreads. The use of three different incubation periods increased the odds of producing good slides due to the temporal variability of division from culture to culture. The best area on the slides were marked with a diamond pencil to the size of the coverslips that were used (22 mm x 22 mm). The slides were then stored frozen and used within a week of their preparation.

Preparation of slides for hybridization:

(4) *RNase Step*

The slides were first treated with RNase, by placing them in a coplin jar containing 100 μg/ml of RNase in 2 X SSC. They were incubated in this solution at 37°C for 60 minutes. The slides were then removed and washed in 2 X SSC three times for 2 minutes each. The slides were dehydrated through consecutive treatments with ice cold 70%, 95% and 100% ethanol (2 minutes each). Slides were allowed to air dry.

(5) *Denaturation Step*

The denaturing solution (70% formamide/ 2 X SSC) was preheated in a coplin jar to 70-72°C. The slides (maximum of two at a time) were then put into the denaturing solution for precisely two minutes. The slides were dehydrated as before, then dried at room temperature. The slides were preheated to 37°C just prior to hybridization.

Preparation of cosmid probe for hybridization:

(6) *Biotin labelling*

Approximately 1.5 μg of cosmid DNA was labelled with biotin using either the Oncor nick translation kit (11-dUTP) or the BRL nick translation kit (14-dATP). Both kits incorporated a biotinylated nucleotide into the probe via nick translation. After the reaction was stopped with EDTA (final concentration of 25 mM) the probe was in a final concentration of approximately 10 ng/μl.

(7) *Preannealing*

Approximately 100 ng of probe was aliquoted into a 0.5 ml tube, to which was added approximately 10-15 μg of COT-1 suppressor DNA (Gibco/BRL). Both cosmid and competitor DNAs were precipitated by addition of 1/10th volume of 3 M sodium acetate pH 4.5 and 2 volumes of 95% ethanol. The mixture was placed at -70°C for 30 minutes, then the DNA was pelleted by centrifugation (14,000 x g) for 10 minutes. The pellet was washed with 70% ethanol, dried and resuspended in the hybridization solution (50% formamide/ 2 X SSC/ 10% dextran sulfate) to a total volume of 14 µl. The pellet was resuspended by repipetting regularly over a two hour period while being incubated at 37°C. The probe/suppressor hybridization mixture was placed in a 75-80°C water bath for 5 minutes to denature the DNA. The probe mixture was then transferred to a 37°C water bath to allow preannealing of repetitive sequences. The preannealing took 1.5 to 2 hours depending on the cosmid probe.

Hybridization:

(8) *Addition of probe to slide*

The probe mixture was pipetted onto the marked area of the prewarmed slide and a glass coverslip applied allowing the solution to spread. The coverslip was sealed with rubber cement. Next, the slide was transferred to a humidifying chamber (square plastic petri dish with wet pieces of Whatmann 3MM filter paper at the bottom, and a raised platform on which to place the slides) at 37°C. The slide was incubated for 16 hours.

Washing and detection:

(9) *Post-hybridization washes*

After the hybridization was complete the rubber cement was carefully removed from the coverslip and the slides were put through the following washes at 45°C with gentle agitation; 50% formamide/ 1 X SSC (2 x 15 minutes), 1 X SSC (2 x 10 minutes), 0.1 X SSC (1 x 5 minutes). The final wash was with 0.1 X SSC at room temperature for 5 minutes. The slides were then transferred to 1 X PBD (Oncor) for two minutes.

(10) *Detection*

Excess solution was briefly blotted, and not allowing the slide to dry, 70 µl of blocking agent (3% high grade BSA from BRL/ 4 X SSC) was added to the slide and a large plastic coverslip placed over it (Oncor). The slide was incubated for 10 minutes at room temperature. The coverslip was then removed, 70 µl of avidin-FITC (Oncor) was added, and the coverslip was placed back onto the slide. The slide was transferred to a humidifying chamber at 37°C for 20 minutes. The slide was then washed in 1 X PBD at 45°C (3 x 5 minutes).

(11) *Amplification*

Next, 70 μl of goat serum (Sigma, 1:10 dilution in 4 X SSC) was added and the slide was incubated at room temperature for 10 minutes with a plastic coverslip. The coverslip was then removed, excess solution tipped off, and 70 μl of anti-avidin FITC antibody (Oncor) was added. Incubation followed at 37°C for 20 minutes. The slide was then washed (3 x 5 minutes) in 1 X PBD at 45°C. The slide was treated with 70 μl of blocking agent and incubated at room temperature for 5 minutes. The solution was tipped off, 70 μl of avidin-FITC was added and the slide was incubated for 20 minutes at 37°C. The slide was finally washed with 1 X PBD (3 x 5 minutes) at 45°C.

Visualization:

(12) *Counterstaining*

The excess PBD was tipped off and 18 μl of propidium iodide with antifade (Oncor) was added. A glass coverslip was placed onto the marked hybridization area (22 mm x 22 mm) and the counterstain allowed to spread for 2-3 minutes. The excess counterstain was blotted away. The slide was then ready for microscopy and photography.

(13) *Microscopy / Photography*

The slide was viewed with a Zeiss Axiophot photomicroscope with a Zeiss 9 filter (blue excitation from 450-490 nm). The chromatic beam splitter allowed wavelengths of under 510 nm to pass through the objective lens, and a barrier filter blocked wavelengths of under 520 nm from passing through to the eyepiece. Photographs were taken with Kodak Ektachrome 100 HC colour slide film.

Results

The results are subdivided into four sections reflecting the different types of duplications or individuals under study. These sections are as follows:- (I) Marker chromosome study, (II) Interstitial duplication/ partial trisomy study, (III) Ring chromosome study, and (IV) Study of apparent CES cases with normal karyotypes.

(I) Marker chromosome study

This study involved 12 CES cases with supernumerary marker chromosomes. Analysis of these individuals was performed to determine and compare both the size of the duplications associated with the marker chromosomes and the approximate location of the breakpoints associated with marker chromosome formation. This analysis was to provide the initial resolution of the CES critical region and phenotype map.

Clinical and cytogenetic analysis

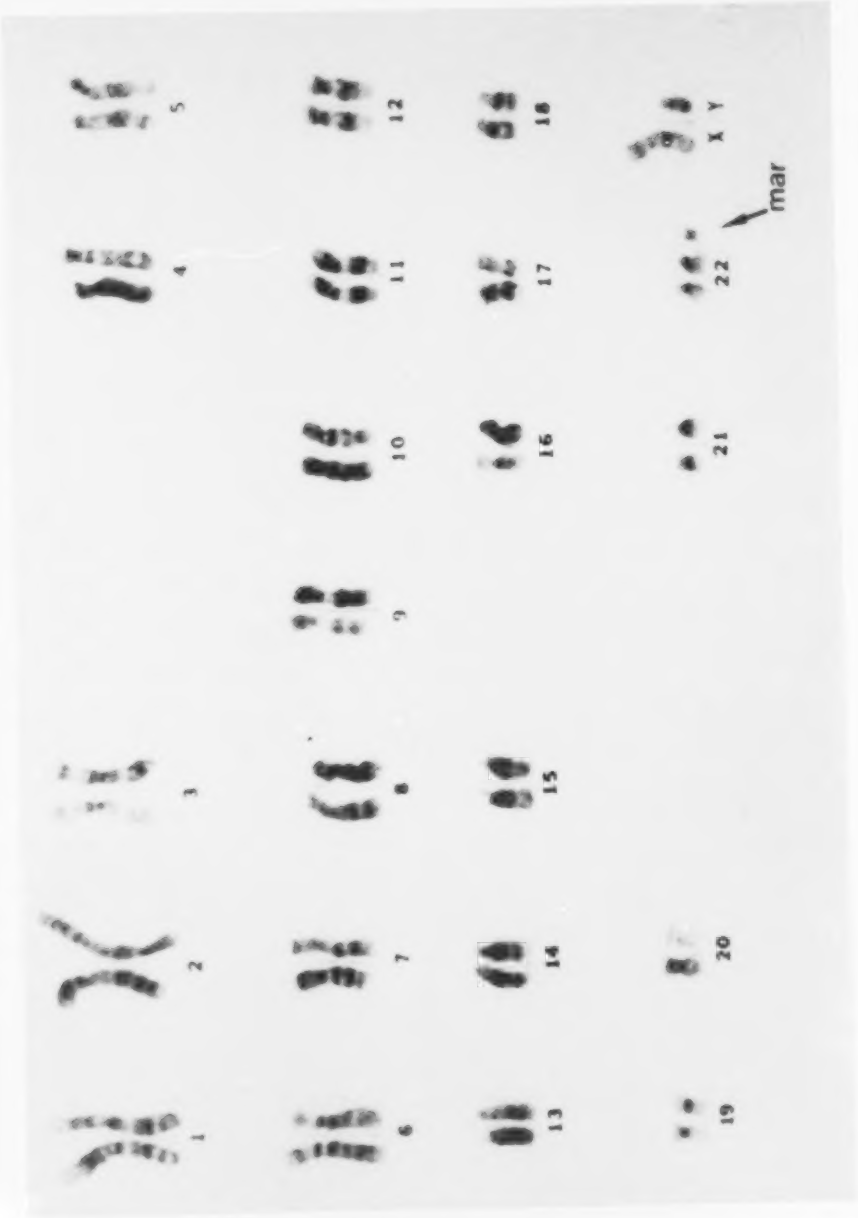
All the patients were referred with a diagnosis of presumed CES because of the presence of some or all of the cardinal phenotypic features of CES (coloboma, skin tags/pits, heart and anal defects). Routine cytogenetic analysis for all 12 patients revealed a supernumerary marker chromosome (karyotype 47, + mar). One example of such a karyotype (for CM11) is shown in Figure 7. CM07 and CM08 are the only related cases in this study (CM07 is the mother of CM08). The 12 patients described show the typical wide-spectrum of phenotypic features, and the major clinical findings are summarized in Table 7.

Dosage Analysis

In order to determine the size of the duplications, DNA was analyzed from all 12 patients by quantitative dosage analysis to determine copy number primarily for the probes to the five loci D22S9, D22S43, D22S57, D22S36 and D22S75. Limited quantitative dosage analysis was also obtained for probes to two other loci, ATP6E and D22S181. An example of part of an autoradiogram (two of seven replicates) used in the

Figure 7 Karyotype of CM11

The G-banded karyotype of CM11 (kindly provided by Dr. B.S. Emanuel) is shown. The supernumerary marker chromosome (mar) is indicated by the arrow.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14
15
16
17
18
19
20
21
22
X
Y
mar

Table 7 Summary of Major Clinical Findings in 12 CES Patients with Marker Chromosomes

	PATIENTS											
	CM01	CM02	CM03	CM04	CM05	CM06	CM07	CM08	CM09	CM10	CM11	CM12
Cardinal features:												
Coloboma	-	-	+	+	+	+	-	-	+	+	+	-
Preauricular tags/pits	+	+	+	+	+	+	+	+	+	?	+	+
Anal defects	+	+	-	+	+	+	+	+	?	?	+	-
Heart defects[a]	-	-	pda	pda	-	tap	-	m	tof	?	vsd	vsd
Developmental status[b]	N	N	?	DD	LN	LN	LN	LN	N	MR	N	DD
Dysmorphic features	+		+		+	+					+	+
Urogenital defects		+		+		+		+			+	
Intestinal anomalies			+			+						
Skeletal defects			+									

A plus sign (+) indicates presence of the phenotypic characteristic; a minus sign (-) indicates absence of the feature. A question mark (?) indicates that no information was available. Ages at developmental classification were as follows: CM01, 4 mo; CM02, 21 mo; CM04, 4 years; CM05, 15 years; CM06, 30 years; CM07, 31 years; CM08, 4 years; CM09, 28 years; CM11, 11 years; and CM12, 15 mo. No information on age was available for CM03 and CM10. Dysmorphic features included down-slanting palpebral fissures (CM01, CM03, CM05, CM06 and CM12), high-bridged nose (CM04 and CM05), epicanthal folds (CM05 and CM06), hypertelorism (CM04, CM05, CM06, CM11 and CM12), micrognathia (CM01 and CM04), and low set posteriorly rotated ears (CM12). Urogenital defects noted were undescended testis (CM02), small genitalia (CM04, CM06, and CM08), absent kidney (CM06), and urethral reflux (CM11). Intestinal anomalies were Hirschsprung disease (CM03) and malrotated gut (CM06). The only skeletal defects identified were Wormian bones and large fontanels (CM03). A blank space indicates that the presence or absence of the feature was not reported in the clinical information.

[a] Heart defects are classified as follows: pda = patent ductus arteriosus; tap = total anomalous pulmonary venous return; tof = tetrology of fallot; m = presence of murmur but no specificied defect; vsd = ventricular septal defect.

[b] Developmental status is classified as follows: N = normal development; LN = low-normal range in IQ; DD = developmental delay; and MR = mental retardation.

dosage analysis of the loci D22S57 and D22S36 for three patients, is shown in Figure 8. The normalized ratios (test probe signal/reference probe signal) for all seven replicates of the autoradiogram partially depicted in Figure 8 are given in Table 8. The statistical analysis for copy number determination from these data sets is shown in Tables 9 and 10. The dosage analysis results for all patients and loci are summarized in Table 11. It was assumed that all individuals have two, three or four copies of each locus investigated.

DNA samples were analyzed for the polymorphic probes to D22S43, D22S57, D22S181, D22S36 and D22S75. Copy number was determined for heterozygous individuals for the respective probes. All 12 individuals were informative for D22S43, and the ratio of allele signal intensities was calculated and compared with those of the disomic and trisomic heterozygous controls, to approximate copy number. Figure 9(a) shows a composite autoradiogram of Southern blots for seven of the genomic DNAs digested with TaqI and probed with D22S43, which shows three alleles. CM01, CM05, CM09 and CM11 were heterozygous with two alleles of approximately equal band intensity, suggesting an even copy number (two or four). CM03, CM04, CM07, CM10 and CM12 were heterozygous with two alleles of unequal band intensities indicating three or four copies. The allelic signal ratios were significantly greater than that obtained for the trisomic control (2:1), indicating four copies for this locus (3:1). CM02, CM06, and CM08 possessed all three alleles, and, in all three cases, two of the allele bands were of equal intensity, and the third was approximately two-fold greater, indicating a total of four copies at this locus (2:1:1).

Five individuals were heterozygous for the polymorphic alleles produced by BstXI and detected by D22S57. CM03 and CM04 showed allele bands of equal intensity (two or four copies). CM06, CM09 and CM12 were heterozygous, with two alleles of unequal band intensities demonstrating a 3:1 ratio, and hence four copies.

Five individuals were heterozygous for the TaqI polymorphic alleles detected by D22S181, with CM09 and CM10 having two or four copies of this locus. CM03, CM06, CM07 and CM12 were shown to possess four copies of D22S181.

Only two individuals were heterozygous for the MspI polymorphic alleles of D22S36. Both CM08 and CM10 demonstrated an even copy number of alleles.

Three individuals were heterozygous for D22S75. CM06 and CM08 possess an even copy number of alleles, whereas CM02 displayed a 2:1 ratio (as compared with the heterozygous trisomic control DNA), indicating three copies of this locus. Figure 9(b) shows an autoradiogram of a Southern blot of TaqI digested genomic DNAs of controls and CM02, CM06 and CM08 after probing with D22S75. The trisomic control allelic

Figure 8 Autoradiogram used for dosage analysis to determine the copy number of the loci D22S36 and D22S57 for CM04, CM05 and CM03.

The composite autoradiogram shows two replicate sets (out of seven in total) from a Southern blot of HindIII- digested DNA from a normal control GM03657, trisomy control GM07106, and three CES patients- CM04, CM05 and CM03. The blot was hybridized with the reference probe to locus D21S110 (3.0 kb), and the test probes to the loci D22S36 (1.0 kb) and D22S57 (0.7 kb). Standardized signal ratios were calculated (test probe signal / reference probe signal) for the complete autoradiogram (data is shown in Table 8 and analyzed in Tables 9 and 10). The three patients show four copies of the D22S57 locus. Patients CM03 and CM04 are in three copies for D22S36, and CM05 has two copies of D22S36.

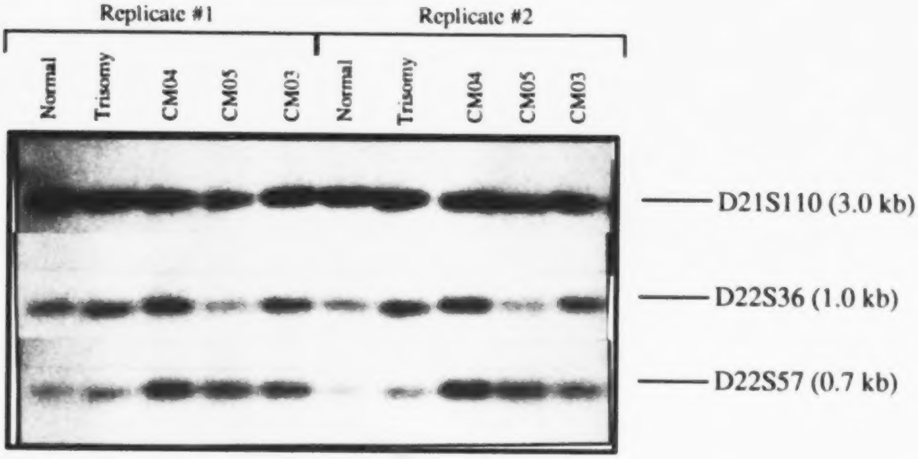

Replicate #1
Replicate #2
Normal
Trisomy
CM04
CM05
CM03
Normal
Trisomy
CM04
CM05
CM03
D21S110 (3.0 kb)
D22S36 (1.0 kb)
D22S57 (0.7 kb)

Table 8 Examples of Data from Dosage Analysis

	NORMALIZED RATIOS FOR THE TEST PROBE D22S57				
RANKING	Disomic Control	Trisomic Control	CM04	CM05	CM03
1	0.25	0.41	0.68	0.60	0.53
2	0.28	0.41	0.68	0.63	0.56
3	0.28	0.42	0.72	0.68	0.61
4	0.33	0.43	0.77	0.71	0.65
5	0.34	0.49	0.79	0.78	0.66
6	0.35	0.50	0.81	0.81	0.69
7	0.36	0.53	0.94	0.83	0.72
Average =	0.31	0.46	0.77	0.72	0.63

	NORMALIZED RATIOS FOR THE TEST PROBE D22S36				
RANKING	Disomic Control	Trisomic Control	CM04	CM05	CM03
1	0.58	0.78	0.74	0.53	0.81
2	0.60	0.79	0.79	0.57	0.82
3	0.66	0.85	0.90	0.64	0.91
4	0.68	0.89	0.96	0.66	0.91
5	0.68	0.90	0.98	0.69	0.95
6	0.73	0.95	1.08	0.70	0.96
7	0.78	0.98	1.14	0.72	1.00
Average =	0.67	0.88	0.94	0.64	0.91

This represents the complete data sets (7 ratios per data set) derived from the autoradiogram which is shown, in part, in Figure 8. The normalized ratios for the data sets are ranked from smallest to largest (top to bottom). The averages for each of the data sets are shown only for comparison purposes and are not used in the statistical analysis of the data.

Table 9 Statistical analysis: Copy number determination of the D22S57 locus for CM04, CM05 and CM03

A

Rank	Disomic	Trisomic	Rank
1	0.25	0.41	8.5
2.5	0.28	0.41	8.5
2.5	0.28	0.42	10
4	0.33	0.43	11
5	0.34	0.49	12
6	0.35	0.50	13
7	0.36	0.53	14
28	Sum of Ranks		77
Trisomic > Disomic (P<0.001)			

B

Rank	Trisomic	CM04	Rank
1.5	0.41	0.68	8.5
1.5	0.41	0.68	8.5
3	0.42	0.72	10
4	0.43	0.77	11
5	0.49	0.79	12
6	0.50	0.81	13
7	0.53	0.94	14
28	Sum of Ranks		77
CM04 > Trisomic (P<0.001)			

C

Rank	Trisomic	CM05	Rank
1	0.41	0.60	8
2	0.41	0.63	9
3	0.42	0.68	10
4	0.43	0.71	11
5	0.49	0.78	12
6	0.50	0.81	13
7	0.53	0.83	14
28	Sum of Ranks		77
CM05 > Trisomic (P<0.001)			

D

Rank	Trisomic	CM03	Rank
1.5	0.41	0.53	7.5
1.5	0.41	0.56	9
3	0.42	0.61	10
4	0.43	0.65	11
5	0.49	0.66	12
6	0.50	0.69	13
7.5	0.53	0.72	14
28.5	Sum of Ranks		76.5
CM03 > Trisomic (P<0.001)			

Each box (A, B, C, and D) represents one statistical test of comparison between two data sets via the Wilcoxon Rank Sum test. The data sets are comprised of seven normalized ratios obtained by the calculation:- D22S57 signal / D21S110 signal for the specific genomic DNAs. (Part of the autoradiogram from which these signals were determined is shown in Figure 8). The 14 normalized ratios are grouped and ranked from 1 (smallest ratio) to 14 (largest ratio). The rank values assigned are then summated for each of the data sets resulting in the sum of ranks. The sum of ranks for each of the data sets are then cross-indexed to determine whether the two data sets are significantly different. A probability of 0.05 was used as the level of significance.

Box **A** shows that the trisomic control data set is significantly greater than the disomic control data set as expected. Boxes **B**, **C**, and **D** demonstrate that the patients data sets for CM04, CM05 and CM03 (respectively) are all significantly greater than the trisomic data set, indicating four copies of the D22S57 locus. The statistical tests of patient data sets against the disomic control are not shown (all are significantly greater than two copies).

Table 10 Statistical analysis: Copy number determination of the D22S36 locus for CM04, CM05 and CM03

Each box (A-G) represents one statistical test of comparison between two data sets via the Wilcoxon Rank Sum test. The data sets comprise seven normalized ratios obtained by the calculation:- D22S36 signal / D21S110 signal for the specific genomic DNAs. Part of the autoradiogram from which these signals were quantified is shown on Figure 8. The 14 normalized ratios for each test are grouped then ranked from 1 (smallest ratio) to 14 (largest ratio). The rank values assigned are then summated for each of the data sets resulting in the sum of ranks. The sum of ranks for each of the data sets are then cross-indexed to determine whether the two data sets are significantly different. A probability of 0.05 was used as the level of significance.

Box **A** shows that the trisomic control data set is significantly greater than that of the disomic control data set as expected. Boxes **B** and **C** for patient CM04 demonstrate that CM04 has three copies of the D22S36 locus. Boxes **D** and **E** for patient CM05 indicate two copies of this locus. Boxes **F** and **G** for patient CM03 conclude three copies of D22S36.

Table 10 Statistical analysis: Copy number determination of the D22S36 locus for CM04, CM05 and CM03

A

Rank	Disomic	Trisomic	Rank
1	0.58	0.78	7.5
2	0.60	0.79	9
3	0.66	0.85	10
4.5	0.68	0.89	11
4.5	0.68	0.90	12
6	0.73	0.95	13
7.5	0.78	0.98	14
28.5	Sum of Ranks		76.5
Trisomic > Disomic (P<0.001)			

B

Rank	Disomic	CM04	Rank
1	0.58	0.74	7
2	0.60	0.79	9
3	0.66	0.90	10
4.5	0.68	0.96	11
4.5	0.68	0.98	12
6	0.73	1.08	13
8	0.78	1.14	14
29	Sum of Ranks		76
CM04 > Disomic (P=0.001)			

C

Rank	Trisomic	CM04	Rank
2	0.78	0.74	1
3.5	0.79	0.79	3.5
5	0.85	0.90	7.5
6	0.89	0.96	10
7.5	0.90	0.98	11.5
9	0.95	1.08	13
11.5	0.98	1.14	14
44.5	Sum of Ranks		60.5
CM04 = Trisomic (P>0.10)			

D

Rank	Disomic	CM05	Rank
3	0.58	0.53	1
4	0.60	0.57	2
6.5	0.66	0.64	5
8.5	0.68	0.66	6.5
8.5	0.68	0.69	10
13	0.73	0.70	11
14	0.78	0.72	12
57.5	Sum of Ranks		47.5
CM05 = Disomic (P>0.10)			

E

Rank	Trisomic	CM05	Rank
8	0.78	0.53	1
9	0.79	0.57	2
10	0.85	0.64	3
11	0.89	0.66	4
12	0.90	0.69	5
13	0.95	0.70	6
14	0.98	0.72	7
77	Sum of Ranks		28
Trisomic > CM05 (P<0.001)			

F

Rank	Disomic	CM03	Rank
1	0.58	0.81	8
2	0.60	0.82	9
3	0.66	0.91	10.5
4.5	0.68	0.91	10.5
4.5	0.68	0.95	12
6	0.73	0.96	13
7	0.78	1.00	14
28	Sum of Ranks		77
CM03 > Disomic (P<0.001)			

G

Rank	Trisomic	CM03	Rank
1	0.78	0.81	3
2	0.79	0.82	4
5	0.85	0.91	8.5
6	0.89	0.91	8.5
7	0.90	0.95	10.5
10.5	0.95	0.96	12
13	0.98	1.00	14
44.5	Sum of Ranks		6[illegible].5
CM03 = Trisomic (P>0.10)			

Table 11 Summary of Dosage and RFLP Analyses for Patients with Marker Chromosomes

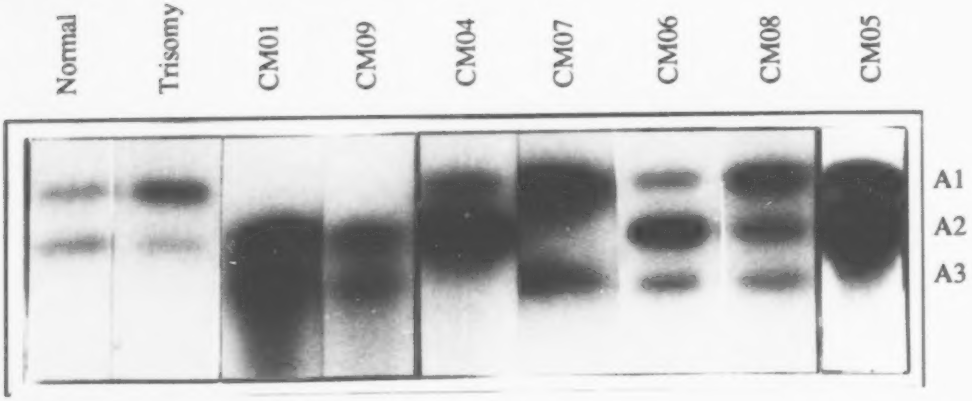
	PATIENTS (CM#)											
Locus and Analysis	01	02	03	04	05	06	07	08	09	10	11	12
D22S9:												
Dosage	4	4	4	4	4	4	4	4	4	4	4	4
D22S43:												
Dosage	4	4	4	4	4	4	4	4	4	4	4	4
RFLP	2/4	4	4	4	2/4	4	4	4	2/4	4	2/4	4
Copy number	4	4	4	4	4	4	4	4	4	4	4	4
ATP6E:												
Dosage	-	-	-	-	-	4	4	4	4	4	4	-
D22S57:												
Dosage	4	4	4	4	4	4	4	4	4	4	4	-
RFLP	NI	NI	2/4	2/4	NI	4	NI	NI	4	NI	NI	4
Copy number	4	4	4	4	4	4	4	4	4	4	4	4
D22S181:												
Dosage	-	-	-	4	4	-	-	4	-	-	4	4
RFLP	NI	NI	4	NI	NI	4	4	NI	2/4	2/4	NI	NI
Copy number	-	-	4	4	4	4	4	4	4	4	4	4
D22S36:												
Dosage	4	3	3	3	2	2	2	2	2	2	3	2
RFLP	NI	NI	NI	NI	NI	NI	NI	2/4	NI	2/4	NI	NI
Copy number	4	3	3	3	2	2	2	2	2	2	3	2
D22S75:												
Dosage	4	3	3	2	2	2	2	2	2	2	2	2
RFLP	NI	3	NI	NI	NI	2/4	NI	2/4	NI	NI	NI	NI
Copy number	4	3	3	2	2	2	2	2	2	2	2	2

NOTE.- The Table shows copy number determined for the seven loci shown to the left. Loci are shown proximal to distal (top to bottom). "-" indicates that analysis was not performed. NI = not informative for RFLP analysis. In the RFLP analysis, the notation "2/4" refers to heterozygotes revealing two bands of equal intensity, indicating an even copy number. Final conclusions on copy number are shown underlined. Values for D22S181 in the dotted boxes indicates that the conclusions were based on findings with FISH analysis.

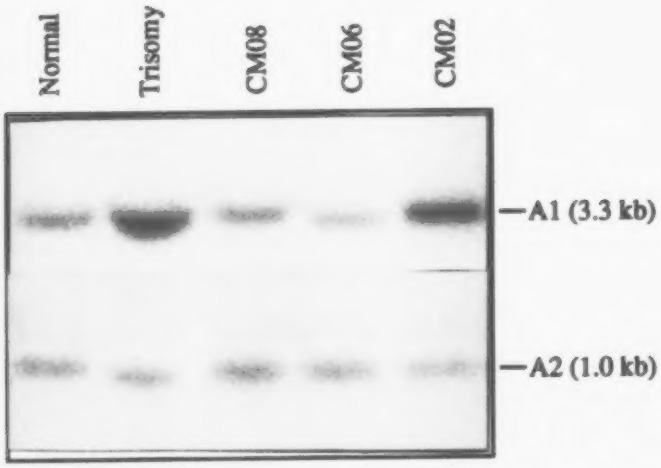
Figure 9 Autoradiograms demonstrating heterozygosity in patients for polymorphic loci D22S43 and D22S75.

(a) Composite autoradiogram of Southern blots of TaqI-digested DNA samples, including normal (GM03657) and trisomic (GM07106) controls and seven of the informative CES patients. The Southern blot was hybridized to the D22S43 probe, that reveals three alleles. Differences in the amount of DNA loaded between lanes precludes direct comparison between lanes. Signal ratios between polymorphic bands in heterozygous individuals were compared with such ratios in heterozygous controls in order to determine copy number. Note that the signal for allele band A2 tends to more intense (20-30%) than the signals for A1 and A3 (corrected in calculations). CM01, CM05 and CM09 show approximate 1:1 signal ratios, indicative of an even copy number (two or four). CM04 and CM07 show signal ratios indicative of a 3:1 distribution (four copies). CM06 and CM08 show an approximate 2:1:1 signal ratio (four copies).

(b) Autoradiogram of a Southern blot of TaqI-digested DNA samples from normal (NC1) and trisomic controls (GM07106), and three heterozygous patients. The Southern blot was hybridized to the D22S75 probe that reveals two alleles. Patients CM08 and CM06 demonstrate approximately 1:1 allelic signals indicative of an even copy number, shown to be two copies by other methods of DNA dosage analysis. Patient CM02 however demonstrates an approximate 2:1 allele signal ratio, indicating three copies of this locus, which was confirmed by other analysis.


Normal
Trisomy
CM01
CM09
CM04
CM07
CM06
CM08
CM05
A1
A2
A3

(a)


Normal
Trisomy
CM08
CM06
CM02
—A1 (3.3 kb)
—A2 (1.0 kb)

(b)

ratios did not differ significantly from the allelic ratios obtained for CM02. The results of the RFLP analysis are summarized in Table 11.

From the complementary methods of DNA dosage analysis performed, the copy number for each patient was determined for five to seven loci. All 12 patients demonstrate four copies of the loci D22S9, D22S43 and D22S57. As would be predicted from the order of the loci, ATP6E is in four copies in all (6/6) patients tested. D22S181 is in four copies in all eight cases determined with DNA dosage analysis. With the FISH analysis described later, two additional patients (CM09 and CM10) are confirmed to also have four copies of this locus. D22S36 is duplicated (three or four copies) in 5/12 patients. D22S75 is in two copies in 9/12 cases.

Fluorescence in situ hybridization analysis

In order to confirm the localization of the D22S36 duplicated sequences on the marker chromosomes, metaphase chromosome spreads from patients CM01, CM03, CM04 and CM11 (CM02 cells were unavailable) were cohybridized with the cosmid probes to the proximal D22S36 test locus (cosS36) and the D22S39 reference locus (cosS39) which maps to the distal tip of 22q. These four patients were previously shown to have duplications of D22S36 by DNA dosage analysis. The cosS36 signal was observed on the marker chromosomes of CM01 and CM03, as expected. However, no cosS36 signal was observed on the marker chromosomes of CM04 and CM11. Instead, it was noted that one of the intact chromosomes 22 in these two patients consistently had greater signal intensity for cosS36, while the distal reference cosmid, cosS39, appeared to be of similar intensity on both homologues. This finding was confirmed by Ms. Beatrice Sellinger in Dr. Beverly Emanuel's laboratory in Philadelphia. In the case of CM04, the two chromosomes 22 could be distinguished from one another on the basis of an observed cytogenetic polymorphism of the short arm. In 84% (21/25) of metaphase spreads examined, the chromosome 22 with the smaller short arm demonstrated a greater fluorescent signal for cosS36. Two metaphase spreads analyzed for CM04 are shown in Figure 10. These results for CM04 and CM11 suggest that, in addition to a marker chromosome, these patients have interstitial duplications encompassing D22S36 on one of the chromosomes 22, an unexpected finding.

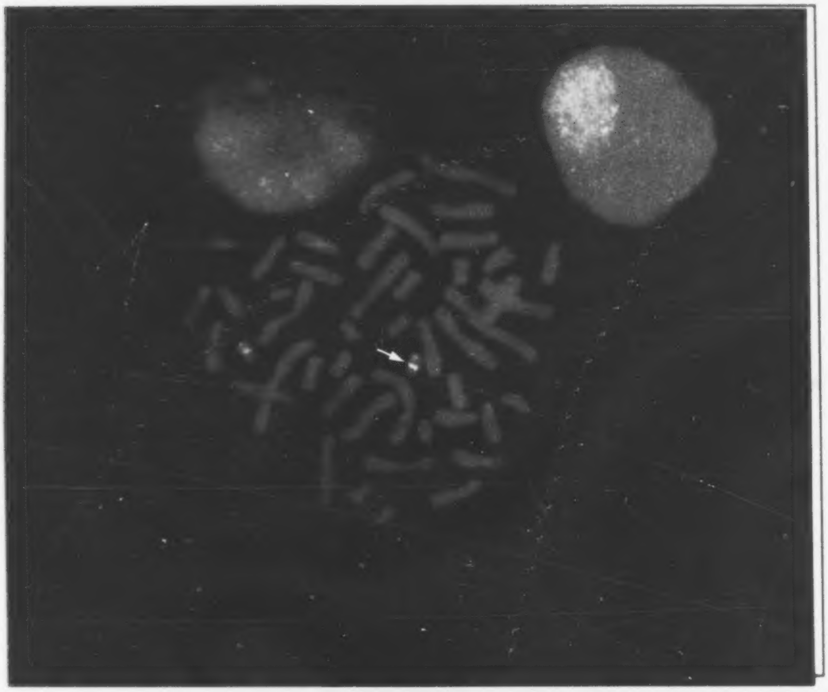
When CM04 and CM11 were cohybridized with the cosmid probe to the D22S181 locus (cosS181) and cosS39, the cosS181 signal was observed on the marker chromosomes, as expected. Thus, only D22S36 indicates the presence of the interstitial duplication. Probe cosS181 also hybridized to the marker chromosomes of all three

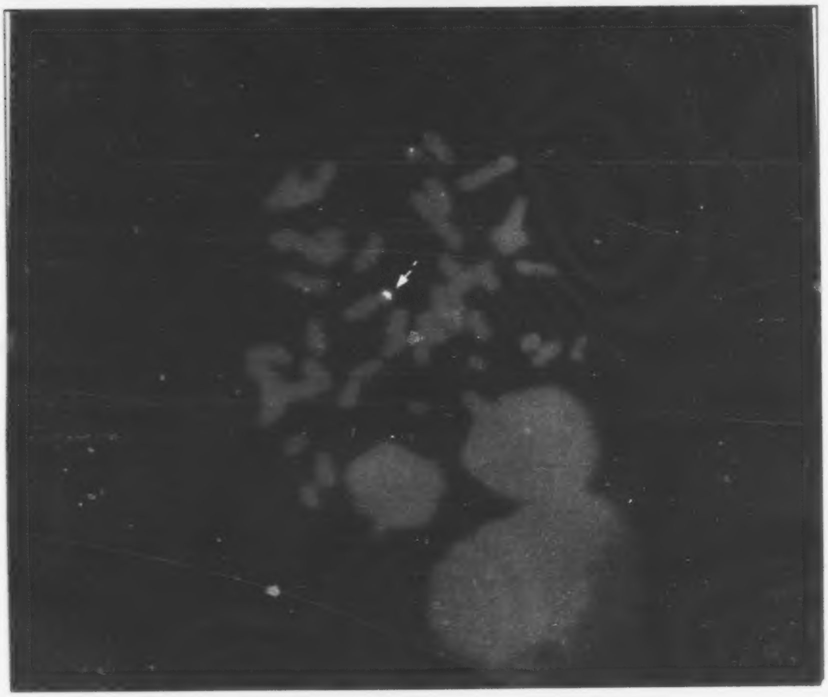
Figure 10 FISH analysis of metaphase chromosomes of patient CM04

(Top) This metaphase spread from patient CM04 was hybridized with the test cosmid probe to the D22S36 locus (cosS36). The chromosomes 22 are identified by the cosmid probe to the D22S39 locus (cosS39) which maps to the distal tip of the long arm. The cosS39 hybridization signals are of comparable intensity, whereas the cosS36 signal is distinctly brighter on one of the chromosomes 22 (denoted by arrow).

(Bottom) This CM04 metaphase spread was hybridized with the cosmid probe cosS36 only, and demonstrates the brighter fluorescent signal on the chromosome 22 with the smaller short arm (denoted by arrow).

Both photographs were kindly provided by Ms.Beatrice Sellinger from Dr.Beverly Emanuel's laboratory in Philadelphia.





other cell lines tested, CM07, CM09 and CM10. Examples of such metaphase spreads are shown for CM09 and CM10 in Figure 11. This finding confirms that the D22S181 locus is in four copies (see Table 11) in two additional patients to the eight identified by DNA dosage analysis described earlier. Thus, the D22S181 locus is duplicated in 10/10 confirmed cases.

D22S36 Interstitial Duplications

From FISH analysis, it was determined that both CM04 and CM11 have interstitial duplications of D22S36 in addition to the presence of a marker chromosome. DNA was available from the karyotypically normal father (YM11) of CM11, and was tested by dosage analysis to determine if he has the D22S36 duplication. Dosage analysis of YM11 confirmed that the father does have a D22S36 duplication. However, neither of the flanking loci are duplicated (D22S181 proximally, D22S75 distally). This confirms the likely origin of the interstitial duplication as paternal. The origin of the marker chromosome in CM11 is uncertain, however, since no maternal sample was available. In the case of CM04, the marker chromosome was also present in the mother, but no blood samples were available for any of the family members and so origin of the interstitial duplication could not be determined.

Parental origins of the marker chromosomes

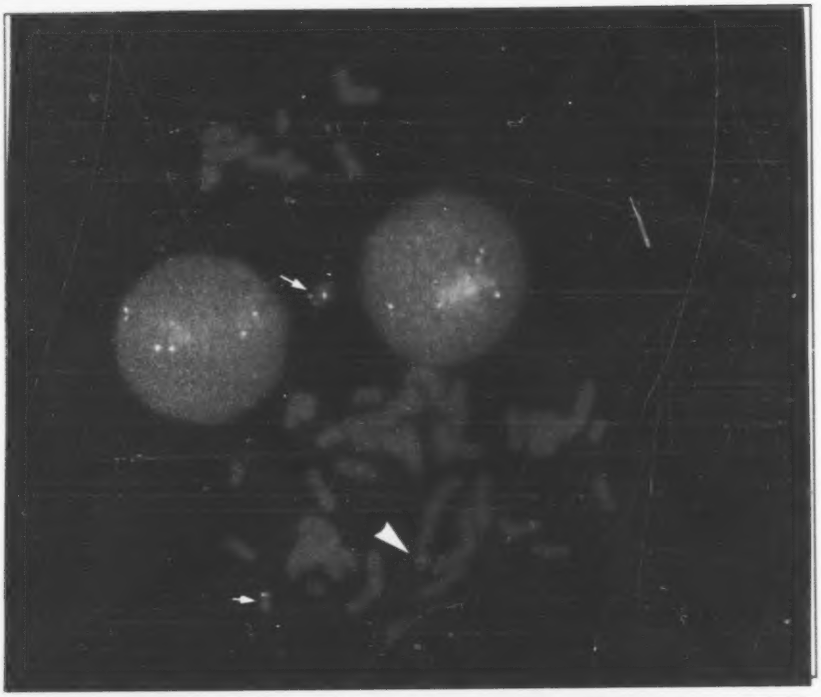
Both paternal and maternal DNA samples were available for CM02 and CM03. From RFLP analysis with D22S43, it can be concluded that in the case of CM02, the marker chromosome is derived from the mother. Figure 12(a) shows an autoradiogram of a Southern blot of genomic DNAs of CM02, the father (YM02) and mother (XM02) digested with TaqI and probed with D22S43. Figure 12(b) shows the pedigree and allelic distribution. YM02 demonstrates an A1 and A3 allele, XM02 an A2 and A3 allele. CM02 has an A1, two A2, and one A3 allele. The A2 allele can only be derived from the mother and is in two copies in CM02, thus the A2 allele is associated with the duplication. Therefore, the duplicated region associated with the marker is derived from the mother. As indicated in Figure 12(c), it is not possible to determine whether the marker is a result of non-sister or sister chromatid recombination.

In the case of CM03, RFLP analysis with D22S57 determined that the CM03 marker is also derived from the mother. Figure 12(d) shows an autoradiogram of a Southern blot of genomic DNAs of CM03, the father (YM03) and mother (XM03)

Figure 11 FISH analysis of metaphase chromosomes of patients CM09 and CM10.

(Top) This metaphase spread from patient CM09 was cohybridized to the cosmid probe to the D22S181 locus (cosS181) and the cosmid probe to the D22S39 locus (cosS39). The chromosomes 22 are identified by the cosS39 signal at the distal portion of the long arm. A cosS181 signal is present on both chromosomes 22 (small arrow), and the marker chromosome (large arrow).

(Bottom) This metaphase spread from patient CM10 was cohybridized to the cosmid probes described above. The cosS181 signal was evident on both chromosomes 22 (small arrow) and the marker chromosome (large arrow).



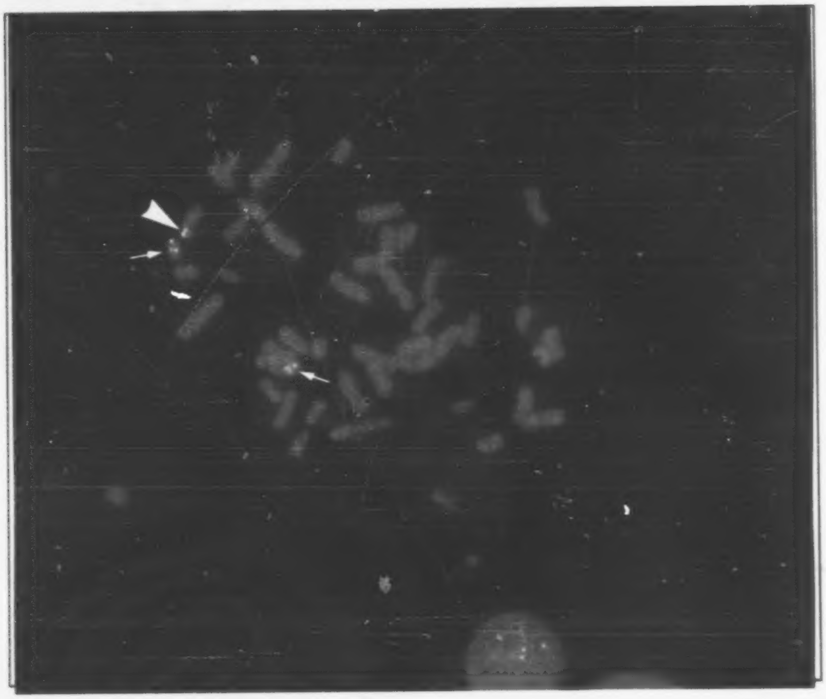


Figure 12 Determination of the the parental origin of the marker chromosomes of CM02 and CM03 with RFLP analysis.

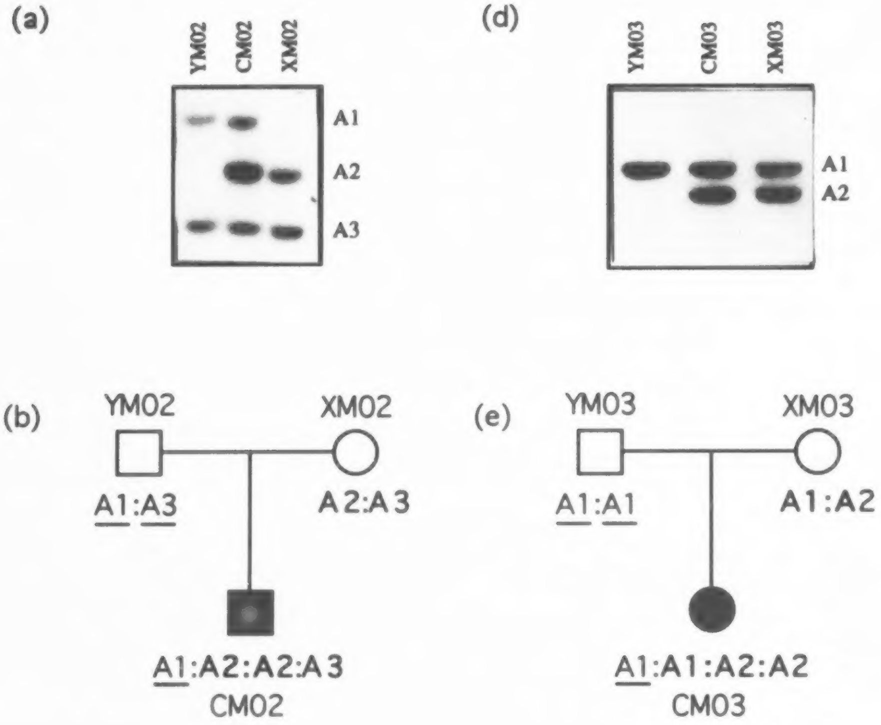
(a) Autoradiogram showing one replicate set from a Southern blot of TaqI-digested DNA from YM02, CM02 and XM02. The blot was hybridized with the probe to the D22S43 locus, which demonstrates polymorphic alleles of 4.8 kb (A1), 3.8 kb (A2) and 2.9 kb (A3).

(b) The pedigree shows the alleles demonstrated by the two parents and the affected child. Paternal alleles are underlined, maternal alleles are in bold. Three alleles of CM02 are maternally derived.

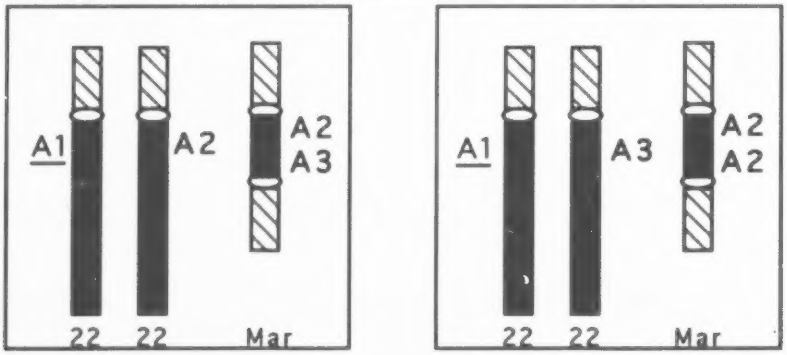
(c) The marker chromosome may be a result of non-sister chromatid exchange (i) or sister chromatid exchange (ii). The possible specific allelic distributions between the chromosomes 22 and the marker chromosome of CM02 are shown for either of these events. Black boxes represent the long arm, and stippled boxes the short arm of chromosomes 22.

(d) Autoradiogram shows one replicate set from a Southern blot of BstXI-digested DNA from YM03, CM03 and XM03. The blot was hybridized with the probe to the D22S57 locus, which demontsrates polymorphic alleles of 2.6 kb (A1) and 2.0 kb (A2). Differences in amounts of DNA loaded between lanes precludes any direct comparison of signal intensity between lanes.

(e) The pedigree shows the alleles demonstrated by the two parents and affected child. Paternal alleles are underlined, maternal alleles are in bold. Three alleles are maternally derived. Involvement of non-sister or sister chromatid exchange in the formation of the marker chromosome of CM03 cannot be determined.


(a)
YM02
CM02
XM02
A1
A2
A3
(d)
YM03
CM03
XM03
A1
A2
(b)
YM02
XM02
A1:A3
A2:A3
A1:A2:A2:A3
CM02
(e)
YM03
XM03
A1:A1
A1:A2
A1:A1:A2:A2
CM03

(c) Possible CM02 Allelic distribution between 22s and marker


A1
A2
A2
A3
22
22
Mar
A1
A3
A2
A2
22
22
Mar

(i) Non-sister chromatid exchange (ii) Sister chromatid exchange digested with BstXI and probed with D22S57. Figure 12(e) shows the pedigree and allelic distribution for D22S57. The father demonstrates two A1 alleles whereas the mother is heterozygous (A1:A2). CM03 demonstrates two A1 alleles and two A2 alleles (equal ratio and four copies). The A2 allele can only be maternally derived, and its presence in two copies in the affected child demonstrates duplication and hence the maternal origin of the marker chromosome. As with CM02, it cannot be distinguished whether a sister chromatid or non-sister chromatid exchange was involved in the marker formation.

A paternal sample only was available for CM11. Both the father (YM11) and son (CM11) were heterozygous for the TaqI polymorphic alleles of D22S43. YM11 demonstrates one A2 allele and one A3 allele, whereas CM11 demonstrates two A2 alleles and two A3 alleles. In the light of this result and the lack of a maternal sample, it was not possible to exclude paternal origin of the marker chromosome with RFLP analysis.

(II) Interstitial duplication / partial trisomy study

This study involved 4 individuals who, from cytogenetic analysis, demonstrated partial trisomy for the 22q11-q12 region. Analysis of these individuals was to investigate whether the associated duplications spanned the complete CES region determined by the marker chromosome study, and if not, whether these duplications further delineate the phenotypic map. Such a comparison between the duplications of these individuals and those studied with the marker chromosomes was particularly relevant in terms of studying the relationship (if any) between dosage of the CES region (three versus four copies) and phenotypic outcome.

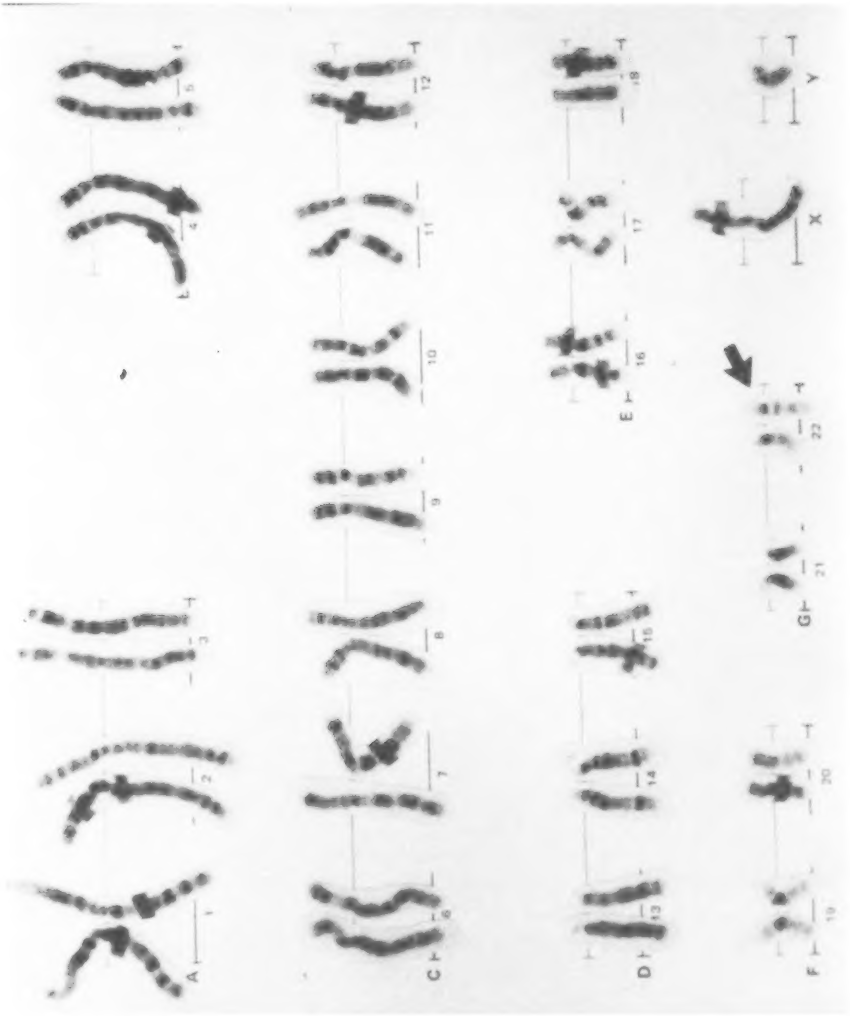
Clinical and cytogenetic analysis

By cytogenetic analysis, CE01, CE02, CE03 and CE04 demonstrated partial trisomy for 22q11-12. The karyotypes for these four individuals are:-

CE01 46, XY, dup (22) (pter-q11.2::q11.1-qter) see Figure 13
CE02 46, XY, dup (22) (pter-q12::q11.2-qter)
CE03 47, XX, +der (22) t(14;22) (q31;q11)
CE04 47, XY, +der (22) t(8;22) (q24.1;q11.2)

Figure 13 G-banded karyotype of CE01

The Giemsa-banded karyotype of CE01 was kindly provided by Dr. Ellen Magenis. The chromosome 22 with the interstitial duplication is denoted by the arrow.


A
1
2
3
4
5
C
6
7
8
9
10
11
12
D
13
14
15
E
16
17
18
F
19
20
G
21
22
X
Y

The two individuals CE01 and CE02 have interstitial duplications, and both demonstrate CES phenotypic characteristics, which are summarized in Table 12. CE03 and CE04 have duplications of 22q11 as a result of unbalanced translocation products. CE03 is clinically normal except for strabismus and was brought to clinical attention due to infertility problems of a sibling with a balanced translocation. CE04 was discovered by amniocentesis. The pregnancy was terminated after 24 weeks but the fetus appeared clinically normal from an extensive post-mortem examination (Dr.W.S.Hwang and Dr.R.Auer, Foothills Hospital, Calgary).

Dosage Analysis

In order to determine the overlap of these partial trisomies with the critical region as defined by the CES marker chromosomes, probes in the region were tested. DNA samples for all four individuals were analyzed for the polymorphic probes to D22S9, D22S43, D22S57, D22S181, and D22S75. Copy number was readily determined for heterozygous individuals, with unequal allelic signals indicating three copies for all the loci in all patients. DNA was also analyzed by quantitative dosage analysis to determine copy number for the non-polymorphic loci D22S795 and D22S543. Analysis was also performed for D22S75 to confirm duplication of this distal locus. Examples of autoradiograms used in dosage analysis are shown in Figure 14. The results of the dosage analysis for these four individuals is summarized in Table 13.

All four individuals are duplicated for all informative tested loci except for CE02 demonstrating only 2 copies of the proximal D22S795 locus. The next most distal locus D22S543 in this individual is duplicated.

Parental origin of the duplications

The duplications of CE03 and CE04, derived from unbalanced translocation products, were both maternally inherited, the mothers being balanced carriers of the respective translocations. No parental samples were available for CE01. Parental DNA samples were available for CE02, and so were tested by RFLP analysis. The one informative polymorphic locus D22S9 enabled determination of the origin of the CE02 interstitial duplication. Figure 15(a) shows an autoradiogram of a Southern blot of TaqI digested DNAs of YE02, CE02 and XE02, hybridized to the probe for the D22S9 locus. CE02 possesses one A1 allele and two A2 alleles. The father is heterozygous (A1:A2), whereas the mother is homozygous for the A1 allele. As shown in Figure 15(b), the two

Table 12 Summary of clinical findings for two CES patients with interstitial duplications

Phenotypic feature	Patient CE01	 CE02
Ocular defects	Bilateral colobomas involving choroid, iris and retina Left micropthalmia	None
Anal defects	None	None
Preauricular malformations	Bilateral preauricular pits	Bilateral preauricular pits
Developmental status	Psychomotor retardation	Moderate motor delay
Congenital heart defect	None	Total anomalous pulmonary venous return
Renal defects	None	Absent right kidney
Urogenital defects	None	Absent right testicle
Dysmorphism	Hypertelorism Flat nasal bridge Downslanting palpebral fissures	Hypertelorism Flat nasal bridge Downslanting palpebral fissures
Other findings	None	Congenital hearing loss

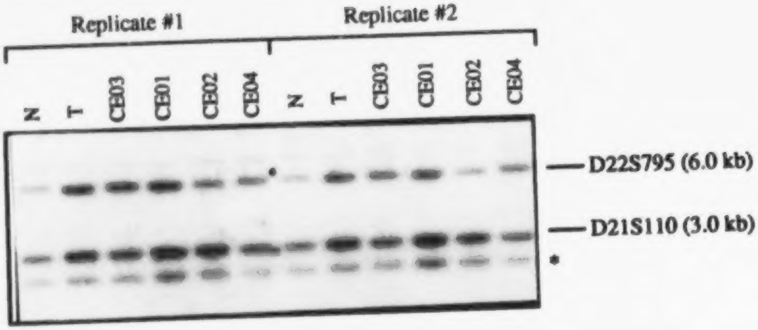
Notes.- Developmental status was determined at age 4.5 years for CE01 and 4 years for CE02.

Figure 14 Dosage and RFLP analysis of proximal (D22S795) and distal (D22S75) loci for cases CE01, CE02, CE03 and CE04

(a) Autoradiogram showing two replicate sets from a Southern blot of HindIII-digested DNA from a normal control (NC1; lane N), trisomy control (GM02325; lane T) and the four partial trisomy cases CE03, CE01, CE02 and CE04. The blot was hybridized with the reference probe D21S110 (3.0 kb) and the test probe D22S795 (6.0 kb). The D22S795 probe shows a homologous band of 2.5 kb (denoted by *), in addition to the 22q11.2 band of approximately 6.0 kb. When standardized signal ratios are calculated (D22S795 signal / D21S110 signal), CE01, CE03 and CE04 demonstrate three copies of this locus. CE02 however, has only two copies of this locus (see Table 13).

(b) Autoradiogram of the same blot described in (a), but after hybridization with the reference probe D21S110 (3.0 kb) and the test probe D22S75 (2.5 kb). In all four cases this locus is shown to be duplicated (see Table 13).

(a)


Replicate #1
Replicate #2
N
T
CE03
CE01
CE02
CE04
N
T
CE03
CE01
CE02
CE04
D22S795 (6.0 kb)
D21S110 (3.0 kb)
*

(b)

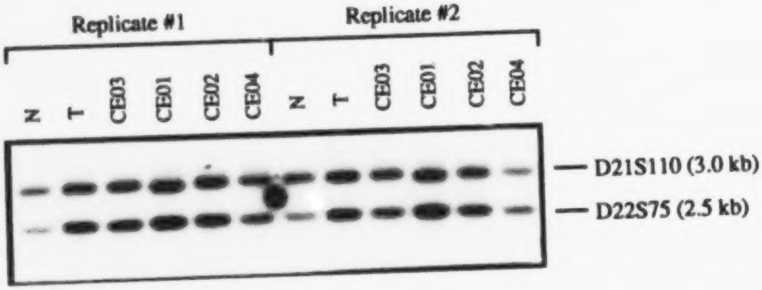

Replicate #1
Replicate #2
N
T
CE03
CE01
CE02
CE04
N
T
CE03
CE01
CE02
CE04
D21S110 (3.0 kb)
D22S75 (2.5 kb)

Table 13 Summary of Dosage and RFLP analysis for individuals with partial trisomy 22

Locus and Analysis	CASE CE01	CE02	CE03	CE04
D22S795:				
Dosage	3	2	3	3
D22S543:				
Dosage	3	3	3	3
D22S9:				
RFLP	NI	3	3	3
D22S43:				
RFLP	NI	NI	3	3
D22S57:				
RFLP	3	-	NI	3
D22S181:				
RFLP	3	NI	3	3
D22S75:				
Dosage	3	3	3	3
RFLP	3	NI	3	3

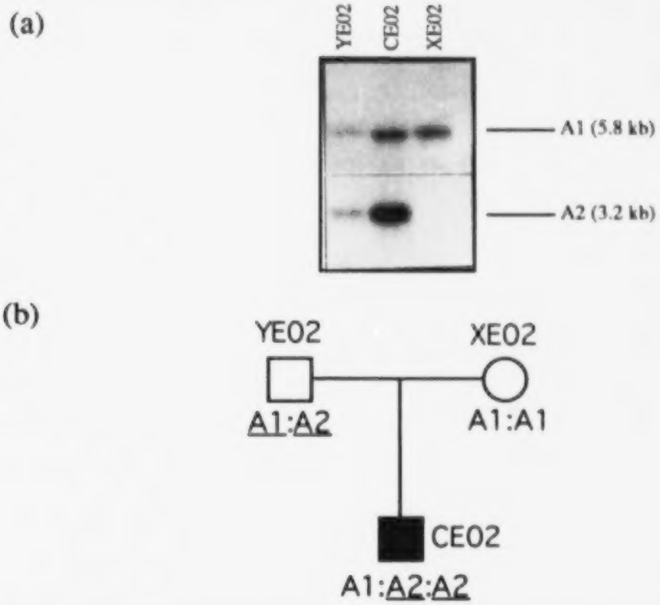
Note.- The table shows the copy number determined for the seven loci shown to the left. Loci are shown proximal to distal (top to bottom). "-" indicates that analysis was not performed. NI = not informative for RFLP analysis. Shading indicates the only case of a non-duplicated locus tested for these four individuals demonstrating partial trisomy.

Figure 15 Determination of the parental origin of the CE02 interstitial duplication using RFLP analysis

(a) An autoradiogram of one replicate set from a Southern blot of TaqI digested DNAs from YE02, CE02 and XE02 is shown. The blot was hybridized with the probe to the locus D22S9. The polymorphic alleles, A1 (5.8 kb) and A2 (3.2 kb) are indicated.

(b) The pedigree illustrates the findings from the autoradiogram, that the father (YE02) is heterozygous (A1:A2) and the mother is homozygous (A1:A1). The paternal A2 allele is duplicated in the affected child and demonstrates the paternal origin of the interstitial duplication. As the duplication is derived from only one of the paternal alleles, sister chromatid recombination is implicated in the formation of this duplication.

(c) One possible model for formation of the duplication of CE02 is shown. In this case, the aberrant recombination event is assumed to occur in paternal meiosis, although mitotic recombination in the paternal germline (formation of spermatogonia) or even in the zygote are also possible mechanisms.


(a)
YE02
CE02
XE02
A1 (5.8 kb)
A2 (3.2 kb)
(b)
YE02
XE02
A1:A2
A1:A1
CE02
A1:A2:A2

Pedigree shows the alleles of the parents and affected child.
The paternal allele (underlined) is duplicated in CE02.

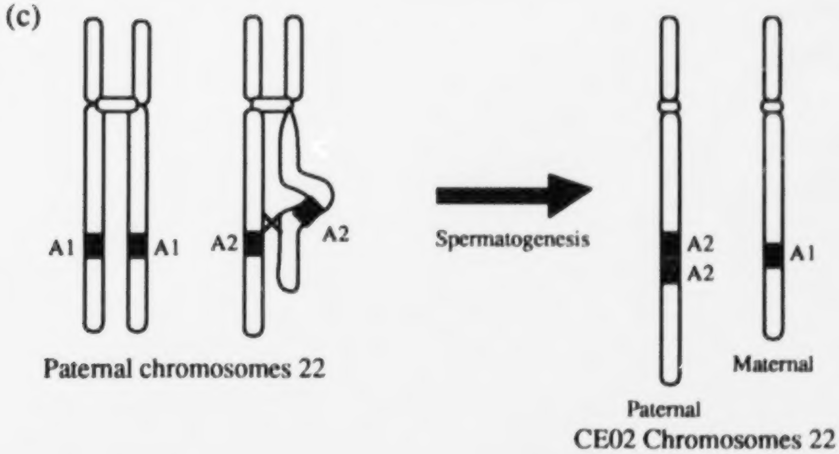

(c)
A1
A1
A2
A2
Spermatogenesis
A2
A2
A1
Paternal chromosomes 22
Maternal
Paternal
CE02 Chromosomes 22

Figure shows a possible aberrant recombination event in paternal meiosis that would result in the formation of the interstitial duplication. The black boxes represent the D22S9 locus.

A2 alleles of CE02 must originate from the father and hence represents the interstitial duplication. In addition, the duplication comprises two A2 alleles, thus the duplication was formed by sister chromatid exchange. Sister chromatid exchange is confirmed with RFLP analysis with the D22S43 locus where the father is heterozygous (A1:A3), the mother homozygous (A1:A1), and CE02 demonstrates three A1 alleles. With the information derived from D22S9 that the duplication is of paternal origin, two A1 alleles are inherited by CE02 from the heterozygous father. Figure 15(c) illustrates a possible mechanism for formation of this interstitial duplication.

(III) Ring chromosome study

This study involved four individuals with supernumerary ring chromosomes derived from duplications of 22q11. Three of the individuals were related and demonstrated familial inheritance of a ring chromosome through three generations. The fourth individual demonstrated de novo occurrence of a supernumerary ring chromosome. Analysis of the duplications associated with these tiny supernumerary ring chromosomes was performed to determine if the duplications would further delineate the critical region and phenotype map. The familial study enabled direct investigation of phenotypic variability with a specific duplication. The approximate localization of breakpoints also enabled comparison with the other duplications to possibly determine if a similar mechanism of formation was involved for these ring chromosomes.

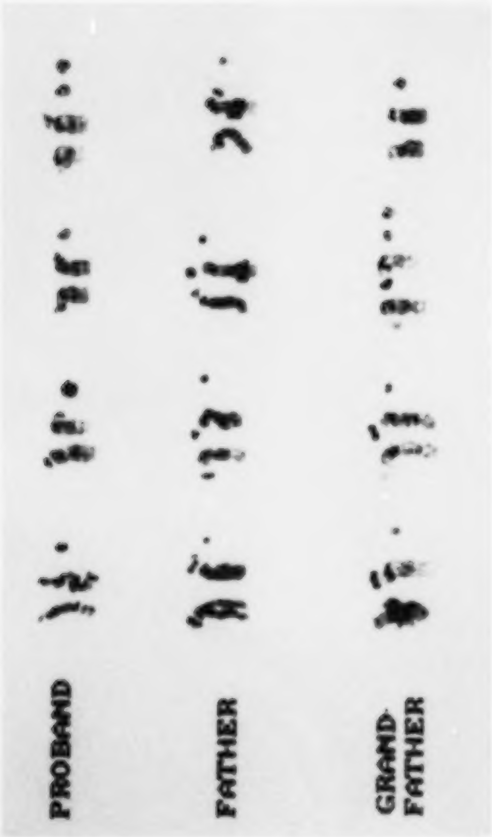
Clinical and cytogenetic evaluation

The proband (CM15) was diagnosed with CES with the following clinical characteristics; coloboma (right), ear pits (bilateral) and tag (left), micrognathia, cleft palate, undescended testes, imperforate anus, total anomalous pulmonary venous return, interrupted aortic arch, ventricular septal defect, atrial septal defect, polycystic kidneys and urethral reflux. The patient died at 17 days.

GTG-banding, C-banding and NOR-silver staining of lymphocyte chromosomes of the proband, and subsequently the father and paternal grandfather showed a supernumerary ring chromosome. Partial karyotypes of all three individuals are shown in Figure 16. The ring in the proband appears larger than that of the father (CM14) and grandfather (CM13). Both CM14 and CM13 were phenotypically normal. The

Figure 16 Partial karyotypes of CM13, CM14 and CM15

Four partial karyotypes showing the chromosomes 22 and the supernumerary ring chromosome are presented for each of the individuals CM13 (grandfather), CM14 (father) and CM15 (proband). The chromosomes are G-banded. Both the size of the ring chromosomes and the number of ring chromosomes may vary as indicated by the partial karyotypes (see Table 14). The supernumerary ring chromosome of the proband is noticeably larger than the one of the father. The partial karyotypes were kindly provided by Dr.Shiva Patil, University of Iowa Hospitals and Clinics.


PROBAND
FATHER
GRAND-
FATHER

cytogenetic findings for blood, skin and lymphoblastoid cell samples for all three family members are summarized in Table 14.

CM16, an unrelated individual, was diagnosed with CES with the following characteristics; preauricular pits, right accessory ear, hypertelorism, low nasal bridge and mild mental retardation. The cell line of this individual demonstrated mosaicism, with approximately 50-60% of the cells having the small supernumerary r(22).

Dosage Analysis

In order to determine the size of the ring chromosomes in comparison to the standard CES marker chromosomes, DNA samples from the cell lines of CM13, CM14 and CM15 were analyzed with the polymorphic probes to D22S9, D22S43, D22S57, and D22S181. Copy number was determined for heterozygous individuals for the respective probes. The cell lines of these three individuals demonstrate only a negligible level of mosaicism and therefore there is no significant effect on dosage analysis.

All three individuals were informative for D22S9. CM13 and CM14 showed a 2:1 allele ratio demonstrating three copies for this locus. CM15 showed a 3:1 allele ratio, demonstrating four copies.

All three were informative for the three allele polymorphic probe to D22S43 (Figure 17a). CM13 showed a 2:1 allele ratio (three copies), CM14 a 1:1:1 allele ratio (three copies), and CM15 a 3:1 allele ratio (four copies).

The next most distal locus ATP6E was tested by quantitative dosage analysis (Figure 17b). A comparison of the ATP6E hybridization signal to the D21S110 reference locus signal demonstrated that CM13 and CM14 have three copies of this locus, and CM15 four copies.

D22S57 was informative for only CM13 and CM14 with both demonstrating a 1:1 allele ratio. Quantitative dosage analysis showed that all three individuals have 2 copies of this locus (Figure 17c). This finding showed that the D22S57 locus maps distally to the ATP6E locus, which could not be determined by physical mapping with pulsed-field gel electrophoresis.

The next most distal locus D22S181 was informative for all three individuals showing a 1:1 allele ratio, indicative of two copies.

A summary of the results is given in Table 15. CM16 was significantly mosaic for the r(22), therefore DNA dosage analysis was not performed.

Table 14 Summary of cytogenetic analysis of blood, skin and cell line samples from CM13, CM14 and CM15

	Blood	Skin	Cell line
Grandfather (CM13)	80/80 (1)	16/20 (0)	50/50 (0)
Father (CM14)	73/80 (1)	10/20 (2)	50/50 (1)
Proband (CM15)	72/80 (9)	NS	50/50 (1)

Numbers represent metaphases studied in which there was at least one ring chromosome observed. The brackets indicate the number of cells in which two rings were observed. NS = not studied. Data from Dr.S.Patil, University of Iowa.

Figure 17 RFLP and dosage analysis of CM13, CM14 and CM15

(a) Autoradiogram showing one replicate set from a Southern blot of TaqI-digested DNA from a normal control (NC1; lane N), a trisomic control (GM07106; lane T) and from three individuals with the supernumerary ring chromosome CM15, CM14 and CM13. The blot was hybridized to the D22S43 probe, which reveals three alleles. Signal ratios between polymorphic bands in heterozygous individuals were compared to heterozygous controls in order to determine copy number. CM13 demonstrated a 2:1 allele ratio (A2:A1), CM14, a 1:1:1 allele ratio (A1:A2:A3), and CM15 a 3:1 ratio (A2:A3). Hence, CM13 and CM14 have three copies of the D22S43 locus, CM15 four copies.

(b) Autoradiogram showing one replicate set of HindIII-digested DNA from a normal control (NC1), a partial trisomy control (GM02325) and from CM15, CM14 and CM13. The blot was hybridized to the ATP6E test probe and the D21S110 reference probe. The ATP6E probe shows multiple homology bands (denoted by *), in addition to the 22q11.2 band of approximately 6.5 kb. The normalized ratios, calculated as the ATP6E signal / D21S110 signal, when compared to the control ratios, demonstrate that CM13 and CM14 have three copies of this locus, and CM15 four copies.

(c) Autoradiogram of the same blot described in (b) except the blot was hybridized to the D22S57 test probe and the D22S44 reference probe. The D22S44 probe maps distal to the duplication of the partial trisomy control cell line GM02325, hence it acts as a reference probe in this situation. The normalized ratios of D22S57 signal / D22S44 signal show that all three individuals have two copies of this locus.

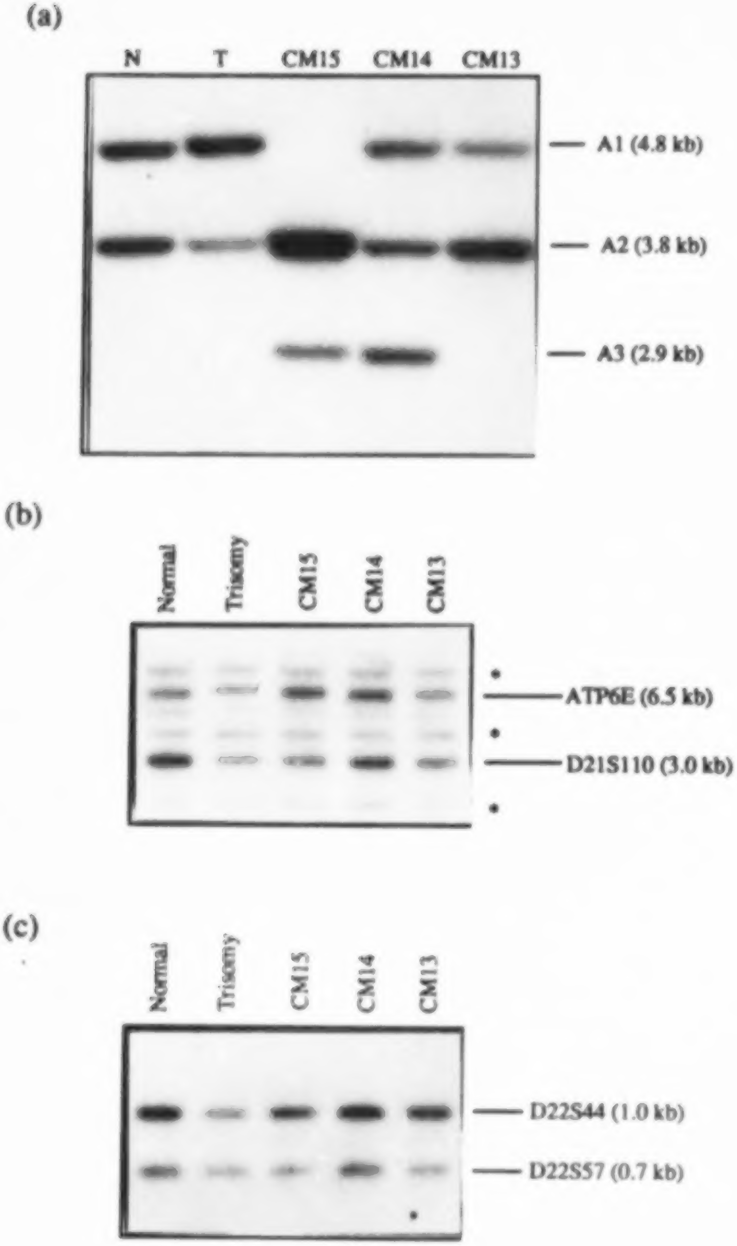

(a)
N
T
CM15
CM14
CM13
A1 (4.8 kb)
A2 (3.8 kb)
A3 (2.9 kb)
(b)
Normal
Trisomy
CM15
CM14
CM13
*
ATP6E (6.5 kb)
*
D21S110 (3.0 kb)
*
(c)
Normal
Trisomy
CM15
CM14
CM13
D22S44 (1.0 kb)
D22S57 (0.7 kb)

Table 15 Summary of Dosage and RFLP Analyses for CM13, CM14 and CM15

	Grandfather	Father	Proband
Locus and Analysis	CM13	CM14	CM15
D22S9			
RFLP................	3	3	4
D22S43			
RFLP................	3	3	4
ATP6E			
Dosage..............	3	3	4
D22S57			
RFLP................	2	2	NI
Dosage..............	2	2	2
D22S181			
RFLP................	2	2	2

Copy number was determined for the five loci shown to the left. Loci are shown proximal to distal (top to bottom). NI = not informative for RFLP analysis.

Fluorescence in situ hybridization

To confirm the localization of the duplicated sequences on the ring, FISH analysis was performed. In CM13,CM14 and CM15, the D22S9 probe signal was present on the ring chromosome. A brighter or multiple signal was often observed on the ring of CM15 (data from Dr.S.Patil at the University of Iowa, and my own observations) when compared to that of CM14 (Figure 18), as predicted from dosage analysis. The ATP6E signal was present on the ring chromosome of CM15. No D22S181 signal was localized to the ring chromosome of CM14.

The ring chromosome of CM16 was tested with the cosmid probes to D22S9 and D22S181. Both these probes demonstrated a signal on the ring chromosome, indicating that this ring chromosome was associated with a larger duplication than that of the other ring chromosome investigated. Due to the limitations of FISH on such a tiny marker, and the mosaicism involved, it was not possible to determine if these probes were in three or four copies in this individual, and as such is not discussed any further.

(IV) Study of apparent CES cases with normal karyotypes

This study involved three individuals with many of the cardinal features of CES but with normal karyotypes. One possibility is that microduplications are present in these individuals resulting in a similar dosage effect observed in the previous three groups studied that can result in CES. Under this premise, DNA dosage analysis with probes from the CES critical region was performed to determine if there were any duplications of these loci in these individuals.

Clinical and cytogenetic evaluation

Three individuals were analyzed who displayed some of the phenotypic features of CES, but had karyotypically normal chromosomes 22. The remaining chromosomes were also apparently normal except for CN01 who is described with the karyotype 46, XX, del (Xq27.1). Note that this deletion is not associated with any CES phenotypic characteristics (Schinzel 1983). The phenotypes of CN01, CN02 and CN03 are summarized in Table 16.

Figure 18 FISH analysis of CM14 and CM15 with the cosmid probe to the D22S9 locus

In both metaphase spreads shown, the test probe is the cosmid for the D22S9 locus (cosS9), and the chromosomes 22 (denoted by small arrows) are identified by the cosmid for the D22S39 locus (cosS39) which produces a signal at the distal tip of the chromosomes 22 . The metaphase spread (A) is of CM14, whereas (B) is from CM15. In both cases a fluorescent signal is clearly observed on the ring chromosome (denoted by large arrow). Furthermore, two clear signals are observed (1/chromatid) on the ring chromosome of CM14, but the ring chromosome of CM15 shows more than two signals indicating two copies of the D22S9 locus on this ring chromosome.

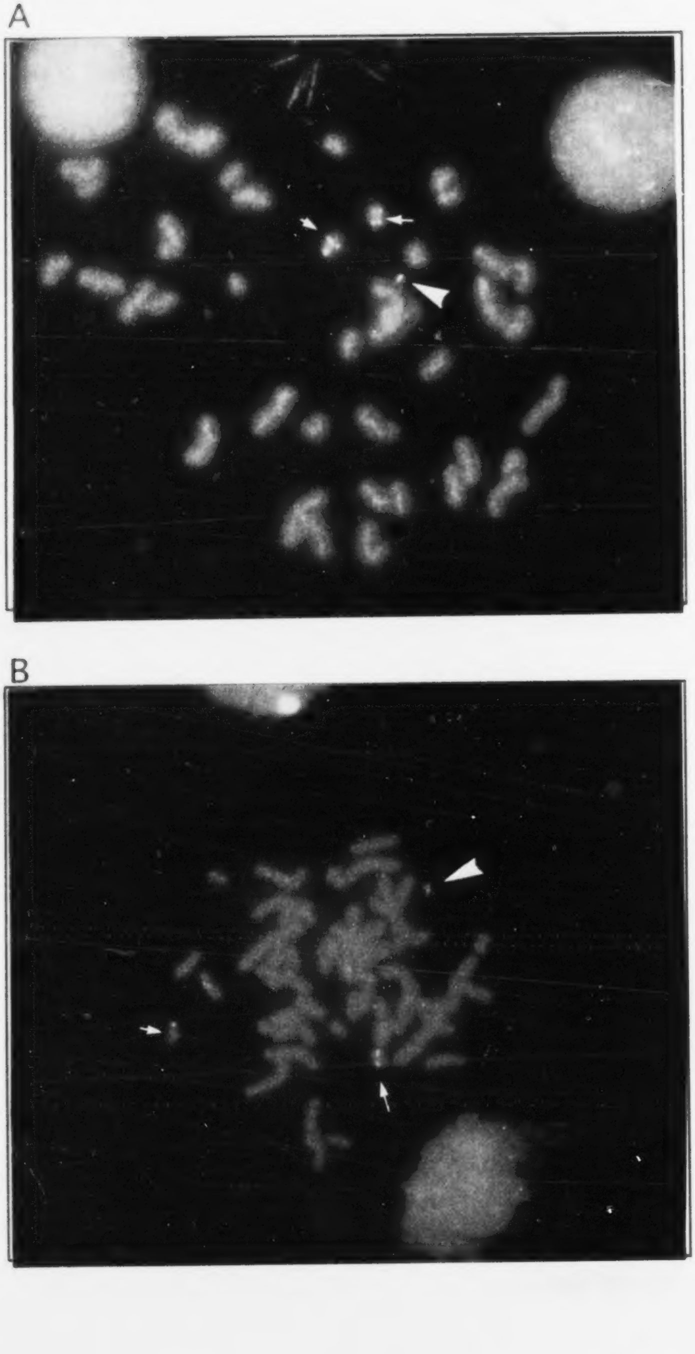

A
B

Table 16 Summary of phenotypic characteristics exhibited by three individuals with apparent CES but a normal karyotype

	Cases		
Phenotype	CN01	CN02	CN03
Ocular defects	Rt.micropthalmia Rt.retinal coloboma Lt.strabismus	Rt.micropthalmia Bl.retinal coloboma	Rt.coloboma
Anal defects	Imperforate anus	Imperforate anus	Imperforate anus
Heart defects	Tetralogy of Fallot	None	None
Renal defects	None	Hydronephrosis	Pyronephritis
Urogenital defects	None	None	Hypospadias Scrotal transposition
Mental Development	Mild retardation	Severe retardation	Normal
Dysmorphism	Downslanting palpebral fissures Depressed nasal bridge	Hypertelorism Small low set ears Microcephaly	Not reported
Other defects noted	Occult spina bifida	Lissencephaly Simian crease	None

Notes.- "Rt" = right, "Lt" = left, and "Bl" = bilateral. Limited reports were available on CN03, especially on dysmorphism. CN03 had a child diagnosed with CES who displayed dysmorphism, bilateral colobomas and micropthalmia, retinal dysplasia, and multiple congenital heart defects which led to death. In addition, a sister of CN03 also has coloboma.

Dosage Analysis

Quantitative dosage analysis was performed for CN01, CN02 and CN03 in order to determine the possible presence of submicroscopic duplications of the CES region. With the loci D22S795, D22S9, D22S43, ATP6E and D22S318, no duplications were detected in these three cases.

Discussion

Characterization of the duplications

The primary goal of this study was to determine the size and structure of the different 22q11.2 duplications, and then to use this information to produce a phenotypic map for CES. Using extensive dosage analysis and limited FISH analysis with probes to 22q11.2 loci, duplications were characterized for twelve individuals with marker chromosomes, four individuals with partial trisomy of the region and four individuals with supernumerary ring 22 chromosomes.

Analysis of the twelve CES cases with marker chromosomes revealed variability in the size of the duplications, but all demonstrated two additional copies (four copies total) of the region spanning the loci D22S9-D22S57 (Figure 19). Two additional copies of the D22S181 locus is also present in all of the markers tested (10/10). Of the two untested marker chromosomes, CM01 is assumed to have two additional copies, and CM02 at least one additional copy of this locus (Table 11). Due to the dicentric structure of the marker chromosomes, the duplications also include the region from D22S9 to the centromere. The locus D22S36 is duplicated in only 5/12 cases and also varies in copy number, being in three copies in four cases (CM02, CM03, CM04 and CM11) and in four copies in only one case (CM01). The locus D22S75 is only duplicated in 3/12 cases (CM01, CM02 and CM03). Thus the marker chromosomes appeared to be subdivided into three different types (Figure 19).

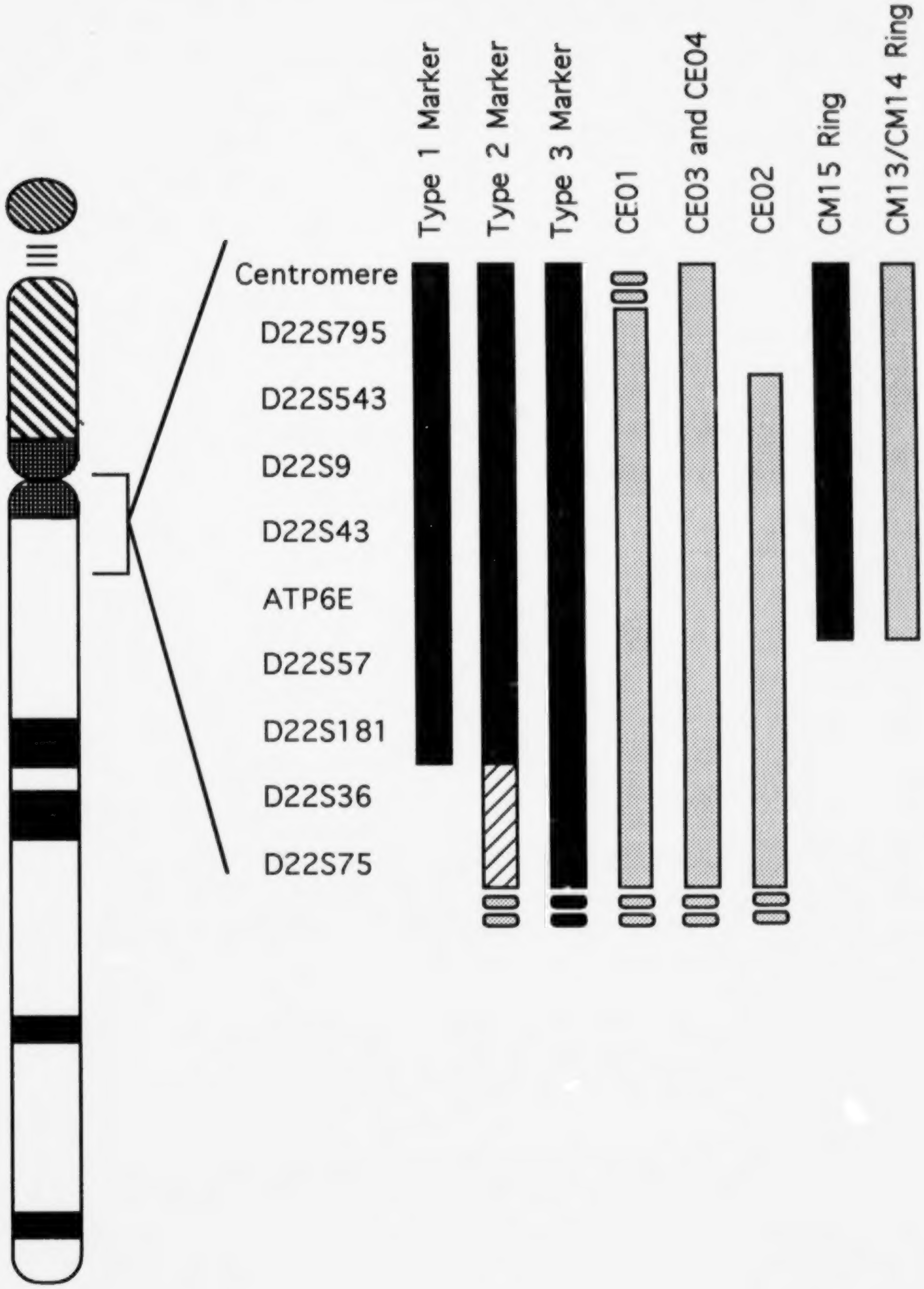
The most common marker chromosome (Type 1) is derived from two duplicated copies of the centromere to D22S181 region, but does not involve duplicated material from the region distal to, and including the D22S36 locus.

The Type 2 marker chromosomes of CM02 and CM03 also comprise two copies of the centromere to D22S57/D22S181 region but in addition have one duplication which extends distally to include the D22S36 and D22S75 loci. These marker chromosomes are therefore asymmetrical and larger than the Type 1 markers. Preliminary data indicates that these duplications extend distal below the DiGeorge region (Kerry McTaggart, personal communication). It is not yet determined whether the distal breakpoint of these two marker chromosomes are the same or differ.

The Type 3 marker chromosome of CM01 is the largest identified (in terms of the loci tested), being derived from two copies of all loci tested of 22q11.2, such that

Figure 19 Summary of 22q11.2 duplications

An ideogram of chromosome 22 is shown to the left. The bracket to the right of the chromosome represents the CES and DGS region. The loci that map to this region which were used in the analysis of the duplications are shown to the right. The extent of the duplications in the individuals studied are shown to the right of the loci. Solid colour indicates four copies, while stippled boxes represent three copies. The individuals or type of duplication is identified above the respective duplication boxes. The small ovals at the ends of certain bars demonstrate that the duplication end-point has not been determined.


Type 1 Marker
Type 2 Marker
Type 3 Marker
CE01
CE03 and CE04
CE02
CM15 Ring
CM13/CM14 Ring
Centromere
D22S795
D22S543
D22S9
D22S43
ATP6E
D22S57
D22S181
D22S36
D22S75

each duplication extends beyond D22S75. This molecular finding corroborates the cytogenetic analysis for CM01 which showed that the marker chromosome was unusually large, being of equivalent size to the G-group chromosomes (Rosenfeld et al. 1984). Most marker chromosomes associated with CES are considerably smaller than the G-group chromosomes (see Figure 7 and Mattei et al. 1984).

By DNA dosage analysis alone, a fourth type of marker chromosome appeared to be evident in the cases of CM04 and CM11, with these individuals having three copies of D22S36, but only two copies of the D22S75 locus. However, with FISH analysis, it was found for both these cases that the duplicated region of D22S36 was not associated with the marker chromosome but represented a small interstitial duplication on one of the "normal" chromosomes 22. Therefore, the actual marker chromosomes of these two cases were of the Type 1 variety. The D22S36 duplicated sequences of CM01 and CM03 (CM02 could not be tested) were confirmed by FISH to be present on the respective Type 3 and Type 2 marker chromosomes. Of the 12 marker chromosomes studied, nine are Type 1 (CM04-CM12), two are Type 2 (CM02 and CM03) and one is Type 3 (CM01).

The four partial trisomy 22 cases, resulting from unbalanced translocation products or interstitial duplications, demonstrated three copies of all the 22q11.2 loci tested except for one case (Figure 19). The interstitial duplication of CE02 did not include the most proximal locus tested D22S795. The second most proximal locus D22S543 is however duplicated. According to analysis of CE02 by Knoll et al. (1995), this duplication extends distally to include the IGLC locus, which is close to the 22q11.2/22q12 interface (McDermid et al. 1993).

The investigation into the familial supernumerary ring chromosome 22 of CM13, CM14 and CM15 demonstrates further variability in the size of duplicated material. The grandfather and father demonstrate three copies of the region spanning the centromere to the ATP6E locus, which is just proximal to the D22S57 locus (Figure 19). This duplicated region spans a maximum distance of approximately 2.1 Mb (Figure 6). The affected child (CM15) had four copies of this region.

Phenotype mapping and definition of the critical region

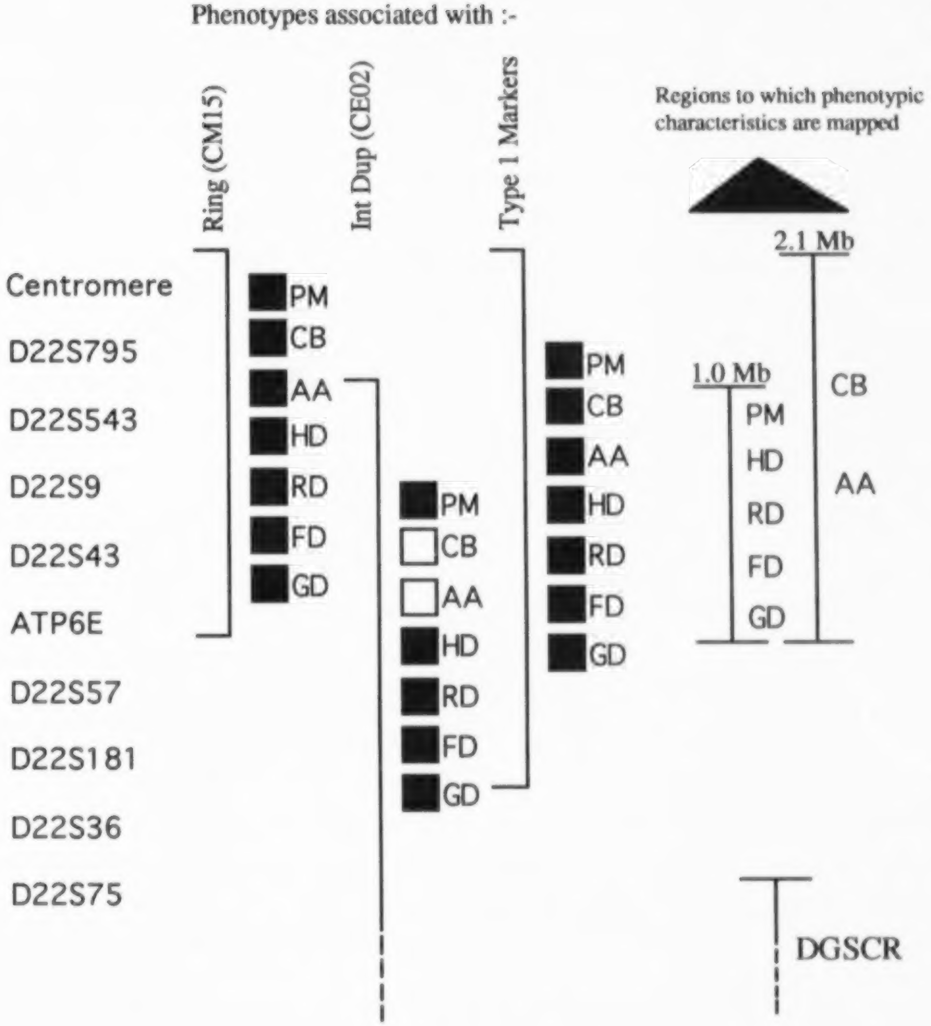
The principles of phenotypic mapping, as outlined by Epstein (1986), are based on the premise that the features of a syndrome resulting from aneuploidy may be correlated to specific segments of that aneuploid region. The "subtraction" method of phenotype mapping was described in detail by Epstein (1986). Basically, if the feature is not present with the aneuploidy of a small segment "A", but is present with aneuploidy of larger region encompassing segment "A" and "B", one may suggest that the region responsible for the phenotypic effect is "B" which constitutes the difference between the two aneuploid states. However, the expression of the phenotypic feature may be a result of region "B" in combination with region "A", i.e. "A" modifies the effect of "B" to produce the phenotype. Thus, to unequivocally implicate segment "B" in the production of the phenotypic feature, aneuploidy for segment "B" alone or as the only common region in different aneuploidies which manifest this phenotypic feature must be demonstrated. Once this is achieved, it is assumed that chromosomal imbalance of segment "B" is responsible (either directly or indirectly) for the phenotypic effect. From such phenotype mapping, the phrase "critical region" has been applied, if somewhat indiscriminately. In general, it refers to the smallest region (or smallest region of overlap) or chromosomal segment, which when imbalanced results in many, all or the more severe phenotypic features of the syndrome. As discussed in the introduction, the phenotype mapping of Down Syndrome (DS) has led to characterization of a "critical region" which maps to the chromosomal segment 21q22.1-qter, which is associated with the more classical phenotypic features of DS such as dysmorphism, heart defect, mental retardation and dermatoglyphics. As is shown by DS, the term "critical region" does not exclude the other regions from having an effect on phenotype. In DS, mental retardation, intestinal anomalies and other features of this syndrome map outside this "critical region".

From the characterization of the duplications described in this study, the phenotypic features of CES can be mapped to regions of 22q11.2. Due to the variability of phenotype observed with CES, as with many other aneuploid syndromes, the lack of a specific feature may either be due to variability (non-penetrance) or due to nonduplication of the gene(s) associated with the phenotypic characteristic. For this reason, as described for DS (Epstein 1986), the CES phenotypic features were only mapped to chromosomal segments of 22q11.2 on the basis of the presence of a characteristic with the presence of the duplicated segment.

From the study of the marker chromosomes, only the distal boundary of the "critical region" could be determined. As all these markers demonstrate duplication of the long arm up to and including the centromere, no proximal boundary could be determined from the analysis of these individuals. Combining the features of the cases of Type 1 marker chromosomes (which represent the smallest duplications) in this study, all the cardinal phenotypic traits of CES were demonstrated (Figure 20). Therefore, the "critical region" (CESCR) to which all CES characteristics may be

Figure 20 Determination of the phenotypic map for CES

This figure shows the key duplications used in the phenotype mapping for CES. The loci are shown from centromere to D22S75. The size of the duplications for the ring chromosome, the CE02 interstitial duplication, and the Type 1 markers are demonstrated by the vertical lines. The corresponding phenotypes (from the combined cases for the Type 1 markers) are shown beside these duplication lines. The seven phenotypic characteristics reported are preauricular malformations (PM), coloboma (CB), anal atresia (AA), heart defects (HD), renal defects (RD), facial dysmorphia (FD), and genital defects (GD). The filled boxes indicate presence of the characteristic, the empty boxes absence. The regions to which the characteristics are mapped and the maximum estimates of their size are shown to the far right, as defined from the duplication and phenotype information. For comparative purposes, the critical region for DiGeorge syndrome (DGSCR) is also shown.


Phenotypes associated with :-
Ring (CM15)
Int Dup (CE02)
Type 1 Markers
Regions to which phenotypic characteristics are mapped
Centromere
D22S795
D22S543
D22S9
D22S43
ATP6E
D22S57
D22S181
D22S36
D22S75
PM
CB
AA
HD
RD
FD
GD
PM
CB
AA
HD
RD
FD
GD
PM
CB
AA
HD
RD
FD
GD
2.1 Mb
1.0 Mb
PM
HD
RD
FD
GD
CB
AA
DGSCR

assigned, maps distal to the centromere and proximal to the D22S36 locus, which spans a maximum distance of approximately 3.3 Mb (Figure 6). The D22S181 locus (duplicated in all the Type 1 markers) is the most distal loci identified within the CESCR as determined by analysis of these marker chromosomes.

The supernumerary ring chromosome 22 of CM15 represents the most significant step in further mapping the CES characteristics (Figure 20). CM15 was clinically described with coloboma, preauricular malformations, dysmorphism, anal atresia, congenital heart defects, renal and genital defects. The only major phenotypic characteristic not able to be determined was mental retardation. This was due to the severely compromising nature of the other congenital anomalies that led to death at only 17 days. For this reason, mental retardation is not included on the phenotypic map of Figure 20. The severe CES phenotype that CM15 displayed was associated with a considerably smaller duplicated region of 22q11.2 (compared to that of the Type 1 marker chromosomes). In this case, the duplication spanned from the centromere to, but not including, the D22S57 locus. This further delineation excludes the D22S57 and D22S181 loci from the CES critical region for nearly all syndromic traits except possibly mental retardation, which could not be assessed. This region spans a maximum of approximately 2.1 Mb which reduces the maximum estimate of the "critical region" by up to 1.2 Mb (Figure 6).

With the ring chromosome of CM15 and the marker chromosomes, the distal boundary of the CESCR was determined. By analysis of the interstitial duplications, the proximal boundary for at least some CES features could also be delineated. The only significant finding with the four cases of partial trisomy 22q11.2 was that the interstitial duplication of CE02 did not include the most proximal locus tested D22S795. However, the phenotypic mapping was not as definitive in this case because this individual did not have two of the major phenotypic features of CES, anal atresia and coloboma. As discussed earlier, the lack of these traits could be due to the nonduplication of the associated gene(s), which would be located between the centromere and D22S795, a region spanning up to 1.0 Mb. Alternatively, this may simply be due to non-penetrance of these characteristics. Indeed 7 of the 59 cases reported in the literature, and CM12 in this study, are missing these features despite the presence of a marker chromosome (Table 1). The other features of CES present in CE02 which include dysmorphism, preauricular malformations and defects of the kidneys, genitalia and heart, may however be mapped distal to D22S795. These characteristics are therefore assigned to the D22S795-D22S57 region, which spans a maximum distance of 1.0 Mb (Figure 6). Although CE02 shows delay in motor skills (assessed at four years of age), mental retardation is not readily mapped distal to D22S795 for two reasons. Firstly, motor delay is not always indicative of mental retardation but rather represents the limits of testing at an early age, and indeed the other health problems of this child may have contributed to this delay in motor skills. Secondly, if the patient demonstrates mental retardation, the interstitial duplication of CE02 extends distally to at least the IGLC locus. Unlike many of the CES features, mental retardation is almost universally associated with aneuploid states (Epstein 1986). With the interstitial duplication of CE02 involving a considerable portion of 22q11.2 distal to D22S75, it is difficult to rule out the additional duplicated region being either directly or indirectly (modifier genes) involved in the expression of motor delay in this patient.

Combining the studies of the duplications of marker chromosomes, ring chromosomes and interstitial duplications, the phenotypic features of CES have been mapped to subregions of 22q11.2, and these are illustrated in Figure 20. Preauricular malformations, heart defects, renal defects, genital defects and facial dysmorphism are all mapped to the region between the loci D22S795 and D22S57. Coloboma and anal atresia map to the region between the centromere and D22S57. At least one factor associated with mental development maps proximal to D22S36. Due to mapping difficulties associated with this trait, mental retardation is not included on the phenotype map of Figure 20.

If the CESCR is defined as the smallest region duplicated which produces all the phenotypic features of CES (excluding mental retardation), then this region is defined by the proximal boundary of the centromere and the distal boundary of D22S57. As such, the CESCR is distinct from the defined critical region for DiGeorge syndrome (DGSCR). Although the D22S36 locus is deleted in some DGS patients, it is not deleted in all cases and thus maps proximal to the DGSCR (Driscoll et al. 1992a). The CESCR defined by this study is proximal to D22S36 and indicates that these two syndromes are not overlapping for any of the phenotypic traits (Figure 20), and do not represent the phenotypic outcomes for reciprocal changes in dosage states of the same genes.

Phenotypic variability

Phenotypic variability is commonly observed with aneuploidies, and it was argued by some that this was a manifestation of the nonspecific effects of chromosomal imbalance. Epstein (1986) cited examples from the literature to support his hypothesis that other factors were involved in the processes that lead to phenotypic variability. Firstly, he noted that in some cases of monozygotic twins, one of the twins would present with a congenital anomaly whereas the other would not. These observations implicated nongenetic factors in the determination of phenotype. Secondly, he argued that phenotypic variability is not unique to aneuploidies which affect large chromosomal segments. Two examples that he presented towards this fact were of single gene defects associated with autosomal dominant diseases such as neurofibromatosis (type 1) and Marfan syndrome, both of which show considerable phenotypic variability, even within the same family (same defect). Finally, the effects of genetic background were demonstrated with a study of the effects of a teratogen, phenytoin, on mice. The effects of this teratogen with *in utero* exposure were quite specific, however the frequency at which these effects were observed varied considerably depending on the inbred line of mice studied. This result suggested that genetic differences in these mice were responsible for the phenotypic variability observed.

With the information described above in mind, Epstein postulated three major factors that could affect the penetrance and expressivity of the phenotypic characteristics associated with aneuploid syndromes. The first factor is of *stochastic influences*. These represent the inherent variability of developmental processes, such as the specific timing of cell divisions. The second factor is *extrinsic* or *environmental* influences. These include the effects of teratogens, maternal anatomy and metabolism. The third factor is that of *genetic background*. The developmental processes involve numerous gene products either directly or indirectly. The variability of these contributing genes may hold the balance of power in terms of the final product, the phenotype. A further consideration lies in the variant alleles of the genes that are affected directly by the aneuploid state. It is possible that the dosage effects vary depending on the specific alleles of the loci that contribute to the phenotypic anomalies. With all this considered, the critical point is that development does not take place in a vacuum, and the combination of direct gene dosage effects with numerous nongenetic and other genetic factors represents a mechanism by which the phenotypic variability may be produced.

In the study of CES, it was assumed that the phenotypic variability observed was due to the effects described above. However, two other possible influences were also investigated that could potentially affect phenotype. The first is the potential effects of modifier loci outside the defined critical region for CES, the second is the effects of the level of dosage on the phenotype, specifically tetrasomy versus trisomy of the CESCR.

The phenotypic mapping defined the CESCR, however, the variability of the size of the duplications raised the possibility that other genes duplicated outside the CESCR in certain marker chromosomes may either play a minor direct role or indirect (modifier) role on the expression of the phenotypic characteristics of CES. In the case of Down syndrome, mental retardation was mapped to several segments of chromosome 21. Although the so-called critical region (DSCR) at the distal portion of 21q was sufficient to result in mental retardation, it was queried whether the other regions of 21 (proximal) to which mental retardation was also mapped, would affect the expression or severity of this characteristic if aneuploid in combination with the DSCR (Korenberg et al. 1992). Although the number of CES cases analyzed in this study is relatively small, a preliminary investigation of potential modifier effects was made on phenotypic severity. Although very subjective, for discussion purposes, severity is loosely determined from the number of major defects that can affect the health and function of an individual, such as heart, anal, and renal defects and mental retardation. The smallest duplications associated with marker chromosomes (Type 1) showed considerable variation in phenotypic severity from mild (CM05, CM07), to very severe (CM06) (see Table 7). The supernumerary ring chromosome of CM15 was associated with an even smaller duplication than that of the Type 1 marker chromosomes and this individual demonstrated one of the most severe (if not the most severe) CES phenotype. The Type 2 and Type 3 marker chromosomes represent larger duplications and although there are only three cases, variability of phenotype is again demonstrated. CM01 shows a very mild phenotype despite having the largest identified duplication. CM02 has a mild phenotype also, however CM03 has a severe CES phenotype. Preliminary analysis would suggest that the phenotype is independent of the size of the duplication beyond the CESCR.

The apparent lack of modifier effects or even novel characteristics with the larger duplications is particularly intriguing in light of the fact that in all three cases (CM01, CM02 and CM03) the duplications extend into the DGSCR. Furthermore, in cases CM01 and CM03 (CM02 not tested), the duplications have been confirmed to involve the complete DGSCR (Kerry McTaggart, personal communication). With the lack of either novel characteristics or particularly severe CES phenotypes (compared with those observed for the smaller duplications) it appears that the DGSCR genes that demonstrate haploinsufficiency, are probably not sensitive to increased dosage states. One gene that has been identified in the DGSCR, TUPLE-1, is assumed to encode a subunit of a multimeric complex, possibly involved in regulation (Halford et al. 1993b). It is postulated that disruption of the stoichiometric balance (50% decrease in one subunit) would affect the function of the complex. One can envisage the situation whereby the decrease of a particular subunit would act as a limiting factor for the amount of functional complex, whereas increases of that subunit would be unlikely to disrupt the amount of complex and hence its function. It is important to note that the number of cases of duplications extending into the DGSCR is small, and failure to note phenotypic effects may be due to non penetrance. Alternatively, such duplications may have an effect which has not been detected. Two possibilities are considered. Firstly, the effects of increased dosage of these gene products may be subtle or currently undetectable. In the case of the reciprocal syndromes of CMT1A (duplication) and HNPP (deletion), the phenotypic effects differed in manifestation and severity, such that they were clinically distinct and considered unrelated until molecular analysis demonstrated otherwise. Secondly, these gene products may modify the severity of the CES phenotype in terms of frequency or severity of specific characteristics. In order to test this hypothesis, a large comparative study of the phenotypes related to different duplications would be required and this is currently not a particularly viable option. Overall in terms of phenotype mapping and variation, problems of phenotypic variability of CES due to modifier effects of regions outside the CESCR are yet to be demonstrated.

Another factor that may play a part in aneuploid syndromes, as discussed by Epstein (1986), is the potential effects of the level of dosage on phenotype. Epstein studied data on the phenotypes of individuals with trisomy or tetrasomy of the same chromosomal segment. Comparison between tetrasomy 9p and trisomy 9p showed few obvious phenotypic differences, though the number of cases was very small. Phenotypic comparisons between tetrasomy and trisomy 18p revealed considerable differences. With the 11 cases of tetrasomy 18p, 21 different phenotypic characteristics were noted. These traits varied in frequency, but the most common of these were psychomotor retardation (11/11), microcephaly (9/11), hypertonia (8/11), and a high-arched or cleft palate (8/11). In stark contrast, the six cases of trisomy 18p demonstrated only 4/21 of the traits, and psychomotor retardation (3/6) was the only representative of the commonest traits mentioned above for tetrasomy 18p. In the case of literature reports of 22q11 for trisomy and tetrasomy (see Introduction), though many phenotypic traits are shared, two traits show particular disparity in frequency between the different aneuploid states. Coloboma has never been observed in individuals with complete trisomy 22 (confirmed) and has only been reported once (Simi et al. 1992) for a duplication resulting from the (11;22) translocation. Anal atresia is considerably more frequent with tetrasomy (75%) than in trisomy cases (20-40%).

This study of CES investigated several individuals that demonstrate trisomy of the CESCR, and as such enables comparison of phenotypes with tetrasomy of the region. One key consideration for any such comparison however is bias of ascertainment. Individuals with duplications (trisomy or tetrasomy) are typically brought to the attention of the clinician on the basis of phenotype. Individuals with a normal or near-normal phenotype may not be investigated cytogenetically unless such analysis is prompted by the birth of an affected child in a family, or if prenatal diagnosis detects a chromosomal anomaly. Thus, a comparison of the effects of trisomy versus tetrasomy can only be discussed in this context of such possible errors of bias.

There are six individuals in this study that have three copies of the CESCR; CE01, CE02, CE03, CE04, CM13 and CM14. CM13 and CM14 possibly represent the most interesting cases for studies into dosage effects. CM13 and CM14 have supernumerary ring chromosomes which are derived from one additional copy of the CESCR region, resulting in partial trisomy. Both these individuals are phenotypically normal. However, the child (CM15) of CM14 demonstrated tetrasomy for the same region due to the doubling of the ring chromosome. This child, unlike CM13 and CM14, demonstrated a very severe CES phenotype. With this familial study, one may reasonably assume that tetrasomy of the CESCR has an effect in terms of penetrance and expressivity of the CES characteristics. In addition to CM13 and CM14, CE03 has a normal phenotype, and CE04 has an assumed normal phenotype on the basis of a fetal autopsy. However, such a simple phenotypic relationship to dosage is disputed by two cases in this study. CE01 and CE02 have CES associated with trisomy of the region. CE01 is relatively mild but does have severe bilateral colobomas (involving iris, choroid and retina). In contrast CE02 presents with many of the CES characteristics but does not have anal atresia or coloboma. It is interesting to note that CM12 has a near identical phenotype to CE02, but the former individual has a Type 1 marker chromosome and four copies of the CESCR. Between the two cases CE01 and CE02, all phenotypic features of CES are expressed except for anal atresia. The duplications of CE01 and CE02 are a result of interstitial duplications whereas CE03 and CE04 have duplications derived from a translocation. Any significance between the types of rearrangements resulting in trisomy and phenotype is unlikely as numerous cases of trisomies from translocations demonstrating some of the CES features have been reported. For example, two cases of CES resulting from unbalanced translocation products are described by Bühler (1972). Furthermore, normal phenotypes have been reported in apparently non-mosaic individuals with typical marker chromosomes (Lüleci et al. 1989) which presumably produce partial tetrasomy for the CES region.

In light of these conflicting observations, what conclusions may be made on the dosage-phenotype relationship in CES? The simple threshold model that may be evident for tetrasomy 18p / trisomy 18p, whereby increases in dosage greatly increase penetrance and expressivity of the associated anomalous traits, is not as clearly implicated for CES. Although the data provided by the supernumerary ring chromosome family supports such a threshold model, there are numerous examples that contradict it. In particular, CE02 shows a considerably more severe phenotype, with an interstitial duplication, than many of the individuals with marker chromosomes (and tetrasomy) described in this study. However, overall, the data collected in this study is in accordance with the trends observed in the literature, which is that tetrasomy 22q11.2 results in a more severe phenotype than does trisomy 22q11.2.

Prenatal diagnosis

The prenatal detection of a supernumerary marker chromosome represents a dilemma for genetic counselors as to the prediction of phenotypic outcome due to difficulties encountered in identification of the origins of that marker chromosome (Benn and Hsu 1984). The identification of marker chromosomes with FISH analysis has greatly improved the odds of informed counseling in these situations. One of the most common origins of marker chromosomes is chromosome 15 (Mattei et al. 1984), and in this case molecular analysis is invaluable as there is a good correlation between size of these marker chromosomes and severity of the phenotype (Cheng et al. 1994).

An example of prenatal diagnosis of CES is in the literature (Reeser et al. 1994). In this case report, the origins of the marker chromosome was confirmed as 22 using the cosmid probe to D22S9 in FISH analysis. Based on the information of increased risk of congenital anomalies, the pregnancy was terminated. Post-mortem examination of the fetus revealed that the only recognizable congenital anomaly was preauricular pits. As is evident in this case, the literature and this thesis, although the identification of marker chromosomes with several cosmid probes to the CESCR is possible, prediction of phenotype is complicated by the extreme variability of phenotype assumed to be due to factors such as genetic background and stochastic influences. This considered, prenatal detection of a 22-derived marker chromosome provides little information towards prediction of phenotypic outcome other than the determination of the associated risks already identified in the literature. Future delineation of the CESCR may enable identification of marker chromosomes that will likely have little to no effect on phenotype, however the identification of defects by ultrasound (except mental retardation) is the only bridging point between phenotypic outcome and predicted phenotype for CES at this time.

Origins of the duplications

Identification of the breakpoints associated with the CES duplications

Formation of the duplications requires physical interaction between certain regions of the chromosomes in breakage or exchange events. With the dosage analysis in this study, the approximate locations of the breakpoints involved in the duplications have been identified. Thus a change in copy number between adjacent loci maps a breakpoint between them.

In the study of the marker chromosomes, eleven of the twelve cases were unrelated (CM07 is the mother of CM08). For the eleven marker chromosomes of assumed independent origin, there are a total of 22 associated breakpoints assuming two breakpoints are required to form each marker chromosome. The majority (80%) of these 22 breakpoints occur between the two loci D22S181 and D22S36, which spans a maximum distance of 1.1 Mb (McDermid et al., submitted). The remaining breakpoints occur distal to the D22S75 locus. Of the eleven markers, only two are confirmed as being asymmetric (CM02 and CM03), having three copies of some of the 22q11.2 loci. Determination of the structure (symmetry vs asymmetry) of the Type 1 markers requires further analysis, as the region within which the breakpoints map is large. Such analysis will also determine if the breakpoints of the different marker chromosomes are similar (i.e. they cluster) or not. Asymmetry indicates the involvement of two distinct regions of chromosome 22 in the formation of marker chromosomes whereas symmetry involves the same region. A comparable study of Cheng et al. (1994) characterized the duplications associated with eleven dicentric marker chromosomes derived from chromosome 15. In the study, three different types of marker chromosomes were identified. All the markers were described as being symmetrical using analysis with eight loci mapped to proximal 15q. However, two asymmetrical markers derived from chromosome 15 have been described in other studies (Nicholls et al. 1989; Shibuya et al. 1991). The breakpoints localized by Cheng et al. (1994) are located in three regions. Two of these regions are very proximal and are associated with the two smaller marker chromosomes identified in 4/11 cases. The third more distal region associated with the largest marker chromosome is observed in 7/11 cases.

The breakpoints associated with the supernumerary ring chromosome 22 of CM13-15 differ from those identified for the marker chromosomes, occurring between ATP6E and D22S57. These two loci are a maximum of 100 kb apart (McDermid et al., submitted). The unrelated supernumerary ring chromosome of CM16 has a different breakpoint from this occurring distal to D22S181. The unique location of the breakpoints of the CM13-15 ring may suggest the involvement of a unique event in contrast to the possible mechanism involved in formation of the marker chromosomes. A possible explanation for this is discussed later.

In the case of the interstitial duplications (CE01 and CE02) of this study, the locations of the proximal breakpoints differ from each other and from the breakpoints previously described. For CE01, the proximal breakpoint maps between the D22S795 locus and the centromere whereas it maps between D22S795 and D22S543 for CE02. The distal breakpoints have not been mapped although previous data (Knoll et al. 1994) determined that the breakpoint of CE02 maps distal to the IGLC locus. The breakpoints associated with the translocations of CE03 and CE04 are distal to the D22S75 locus. Further localization of the distal breakpoints of CE01, CE02, CE03, CE04 and of the larger marker chromosomes (CM01, CM02, and CM03) will prove useful in order to determine if any of these breakpoints map to the same region (cluster) or even to other breakpoint regions mapped to 22q11-12 such as the t(11;22) constitutional breakpoint.

Structure of the breakpoint regions

Many individuals have hypothesized on the possible structure and composition of regions that may contribute to "instability", which manifests as various rearrangements. For chromosome 15, the instability of the proximal region (15q11-15q13) has been demonstrated by numerous rearrangements including deletions (Prader-Willi syndrome (PWS) and Angelman syndrome (AS)) translocations with numerous autosomes and duplications associated with the formation of marker chromosomes (Mattei et al. 1984). Furthermore, the role of the same region in different rearrangements is apparently suggested by the mapping of the deletion breakpoints to the same regions to which breakpoints associated with marker formation have been mapped (Robinson et al. 1993; Cheng et al. 1994). Magenis et al. (1988) noted that proximal 22q was also associated with interstitial deletions (DGS) and duplications (CES), translocations (cancer related and constitutional), and duplications associated with formation of marker chromosomes. For this reason it was proposed that these striking similarities between the two acrocentrics 15 and 22 were due to certain unique properties located in the proximal region of the respective long arms.

The first substantial clue to the possible nature of these "unique properties" was provided by Donlon et al. (1986), who while cloning in the PWS breakpoint region discovered numerous repeat palindromic sequences. This discovery of repeats and their possible connection to the instability of proximal 15q had already been predicted several years earlier (Mattei et al. 1984). The role of repeat sequences in rearrangements is not a new concept. In the literature there are numerous such examples. The neurological diseases of CMT1A and HNPP are caused by aberrant recombination between low-copy repeat sequences which results in duplication and deletion of a 1.5 Mb region of 17p. Unequal crossover between Alu-sequences have been demonstrated to result in deletion of seven exons of the LDL-receptor gene (Lehrman et al. 1987). Deletions of the X-chromosome short arm of up to 2.0 Mb are also due to repetitive elements (Yen et al. 1990) and such rearrangements may occur at surprisingly high frequencies (1 in 10,000 individuals) as demonstrated for the deletions of the steroid sulfatase gene (Bonifas et al. 1987).

As for chromosome 22, considerable evidence (if somewhat circumstantial) is gathering as to the possible role of repeat sequences of chromosome 22 in rearrangements. Cloning of the and BCR-1 region (breakpoint involved in formation of the cancer-related "Philadelphia chromosome") found a surprisingly high number of Alu-repeats with a frequency of one per 1500 bp (Scambler 1994). More specific repeat sequences have also been identified on chromosome 22 such as the KI-386 family (Carey et al. 1990) and the BCR-related and gamma glutamyl transpeptidase sequences (Heisterkamp and Groffen 1988). These sequences have multiple loci on chromosome 22 and in some cases throughout the genome. One of the most intriguing of such low-copy repeats have been reported by Halford et al. (1993a). These repeats, which are unrelated to others previously described, were discovered during cloning in the DGS region, and were found flanking the region. Their role in some of the deletions of DGS has been suggested. Indirect evidence for the presence and role of repeat sequences in the rearrangements of the CES and DGS regions of 22q11 has been provided by the considerable difficulty encountered in attempts to clone both the DGS region (Halford et al. 1993c), and one of the major CES breakpoint region between the D22S57 and D22S36 loci (Marcia Budarf, personal communication). However, despite the growing collection of evidence for a role of repeat sequences, proof requires the cloning of breakpoints to confirm these predictions, and as already indicated, such cloning is highly problematic.

Parental origin

The study of parental origin can provide information on the nature of the possible mechanism involved in the formation of a rearrangement and whether imprinting is involved or not. In this study, parental origin was successfully determined with RFLP analysis, for two of the patients with marker chromosomes and one of the patients with an interstitial duplication.

For the two marker chromosome patients CM02 and CM03, maternal origin is evident. The type of chromatid exchange (sister or non-sister) involved in the formation of these maternally derived marker chromosomes could not, however, be determined. Two other cases of confirmed parental origins in de novo marker formation (Magenis et al. 1988) implicated maternal origin and non-sister chromatid exchange in the formation of the marker chromosome. Maternal origin is also indicated in the majority of inv dup(15) cases (see Introduction). Due to the fact that both the inv dup(15) and (22) marker chromosomes are supernumerary structures, it is assumed that a non-disjunction event must be occurring to result in two normal acrocentrics plus the derived marker chromosome. Due to the fact that maternal origin has been found to be prevalent in cases of aneuploidies resulting from nondisjunction (95% for trisomy 21, Stewart et al. 1988), these findings for marker chromosomes of 15 and 22 are not surprising. However, the formation of the marker chromosomes also requires aberrant recombination or structural rearrangements, which are assumed to be paternally derived in the majority of cases (Olson and Magenis 1987). This considered, the predicted parental origin of the marker chromosome appears to be in somewhat of a conflict, however, preferential maternal origin suggests that nondisjunction is the key event.

In the case of the interstitial duplication of CE02, the parental origin was determined to be paternal and to be the result of sister-chromatid exchange. The paternal origin is consistent with this type of structural rearrangement (Olson and Magenis 1987). The presence of the CES phenotype with an additional copy of the CESCR from the father would suggest that paternal imprinting is not involved in the region.

Previously proposed models of formation of marker chromosomes

Several models have been proposed to explain the formation of the intriguing structures of the inv dup marker chromosomes associated with chromosomes 15 and 22.

Of the various proposed models, two are particularly favored in the literature; (A) paracentric inversions, (B) U-strand exchange.

(A) The paracentric inversion model (Schreck et al. 1977) proposes that a crossover occurs in the inversion loop which forms during synapsis between the normal chromosome 22 and its homolog with the paracentric inversion. The resulting products of such an exchange are an acentric fragment (which is lost) and dicentric fragment. During meiosis I, instead of the theorized breaking of the dicentric (Srb and Owen 1952), nondisjunction would occur with both chromosomes 22 segregating to one pole. In meiosis II, segregation would result in one gamete with a normal or inverted chromosome 22, another gamete with the other chromosome 22 and the dicentric marker chromosome, and the remaining two gametes with no chromosome 22. If the crossover-inversion loop model is correct, the dicentric should be asymmetric with all loci in the original inversion in three copies (two on the chromosomes 22, one copy on the marker), all the loci proximal to the inversion in four copies, and all loci below the inversion in two copies (not duplicated on the marker chromosome). This paracentric inversion model is illustrated in Figure 21. There is some limited evidence for such a model. Firstly, paracentric inversions have been reported for many chromosomes (Fryns et al. 1986). Del Porto et al. (1984) described a paracentric inversion of 15, inv (15)(q15;q24), which resulted in reduced fertility and several neonatal deaths in the pedigree. One theory offered was that the paracentric inversion was responsible for formation of marker chromosomes which due to the resulting extensive duplication of 15, resulted in the spontaneous abortions and deaths within this family. Unfortunately, cytogenetics was not performed to prove or disprove such a theory. In the case of chromosome 22, there have been no reports of paracentric inversions, however, cytogenetically such an inversion of 22q11.2 may be virtually impossible to detect due to lack of discriminatory bands. There is some molecular evidence against this model of marker formation for chromosomes 15 and 22. The paracentric inversion model predicts asymmetry for the marker chromosomes. Within the limits of the methods used for detection, there are very few confirmed cases of asymmetry for 15 (Robinson et al. 1993), and the most recent study of Cheng et al. (1994) found no asymmetric marker in 11/11 cases. For CES, asymmetry has been demonstrated for 2/11 marker chromosomes of independent origin. However, the other 9 have breakpoints within a region spanning over 1.0 Mb and as such require further analysis to confirm the type of structure. At this point however, for both chromosomes 15 and 22, the paracentric inversion model may only represent a rare alternative mechanism by which marker chromosomes are formed.

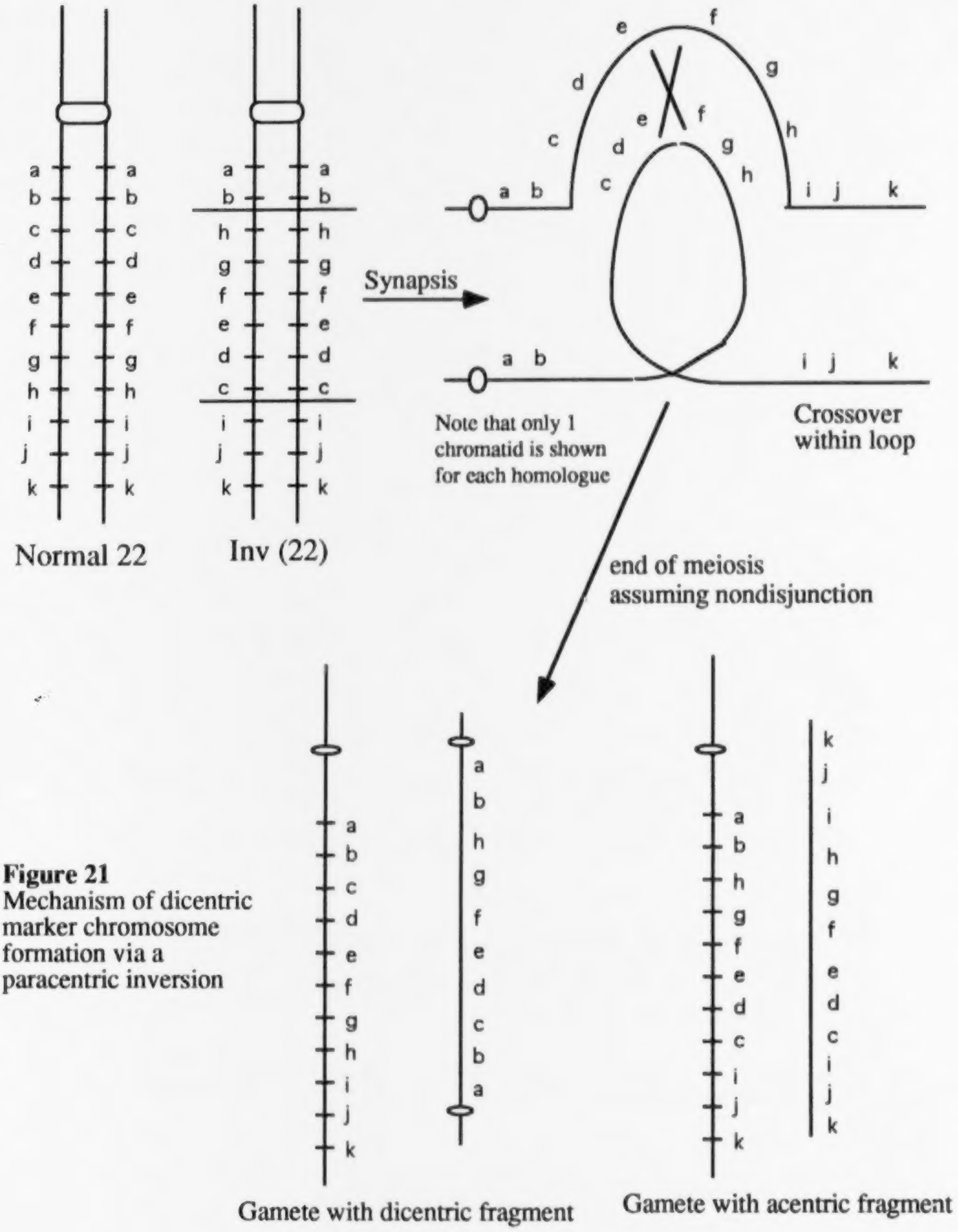

a b c d e f g h i j k
a b h g f e d c i j k
Normal 22
Inv (22)
Synapsis
Note that only 1 chromatid is shown for each homologue
Crossover within loop
end of meiosis assuming nondisjunction
Gamete with dicentric fragment
Gamete with acentric fragment

Figure 21
Mechanism of dicentric marker chromosome formation via a paracentric inversion

(B) The U-strand exchange model proposed by Van Dyke et al. (1977) suggests that there is abnormal breakage and reunion between two homologous non-sister chromatids during parental meiosis I connecting the two centromeres and forming acentric fragments (Figure 22). During anaphase I there is nondisjunction and the homologous chromosomes segregate to one pole leaving one daughter cell without the particular chromosome. During anaphase II, the chromatids segregate producing one normal gamete, with the other gamete containing the one normal chromosome and the dicentric marker chromosome. Asymmetry or symmetry in this case would be dependent on the location of the regions of exchange. The steps of this model of marker chromosome formation are outlined in Figure 22. There is no contradictory evidence against such a model to date and its simplicity is probably its most attractive trait. Such a model does require alignment of sequences that are of opposite orientation, such as repeats.

One key consideration of both these models is the requirement for a nondisjunction event. The complication in marker formation is the fact that a dicentric structure is involved. As originally hypothesized for the paracentric inversions (Srb and Owen 1952) and equally applicable to the U-strand exchange model, one might predict that the dicentric structure would undergo a bridge-break event during segregation due to the antagonistic forces that it must endure after spindle attachment. For both models, it is proposed that such a dilemma is averted due to inactivation of one of the centromeres of the dicentric structure (Schreck et al. 1977; Weleber et al. 1977). Evidence for this is provided by the observation of somatic stability of marker chromosomes (Warburton et al. 1973; Van Dyke et al. 1977). However, such stability is not always observed which results in loss of the marker (see Figure 3) or secondary products resulting from breakage of the marker (Ing et al. 1987; Urioste et al. 1994). The molecular mechanism by which such stability may be endowed, possibly in a transient manner, is currently unknown.

Mechanisms have been proposed to explain the formation of ring chromosomes as are observed with CM13-16 in this study, however, most of these proposed mechanisms result in deletions, not duplications (McGinniss et al. 1992). The popular model for ring chromosome formation involves breaks in the short and long arms followed by fusion. In order to explain the karyotypes observed in this study (two normal chromosomes 22 and a ring), there would have to be a nondisjunction of 22s in one of the parents. These concurrent events are highly unlikely but an alternative may be evident from the literature. Instability of marker chromosomes has been implicated in the formation of secondary derivative products. In Urioste et al. (1994), a family is

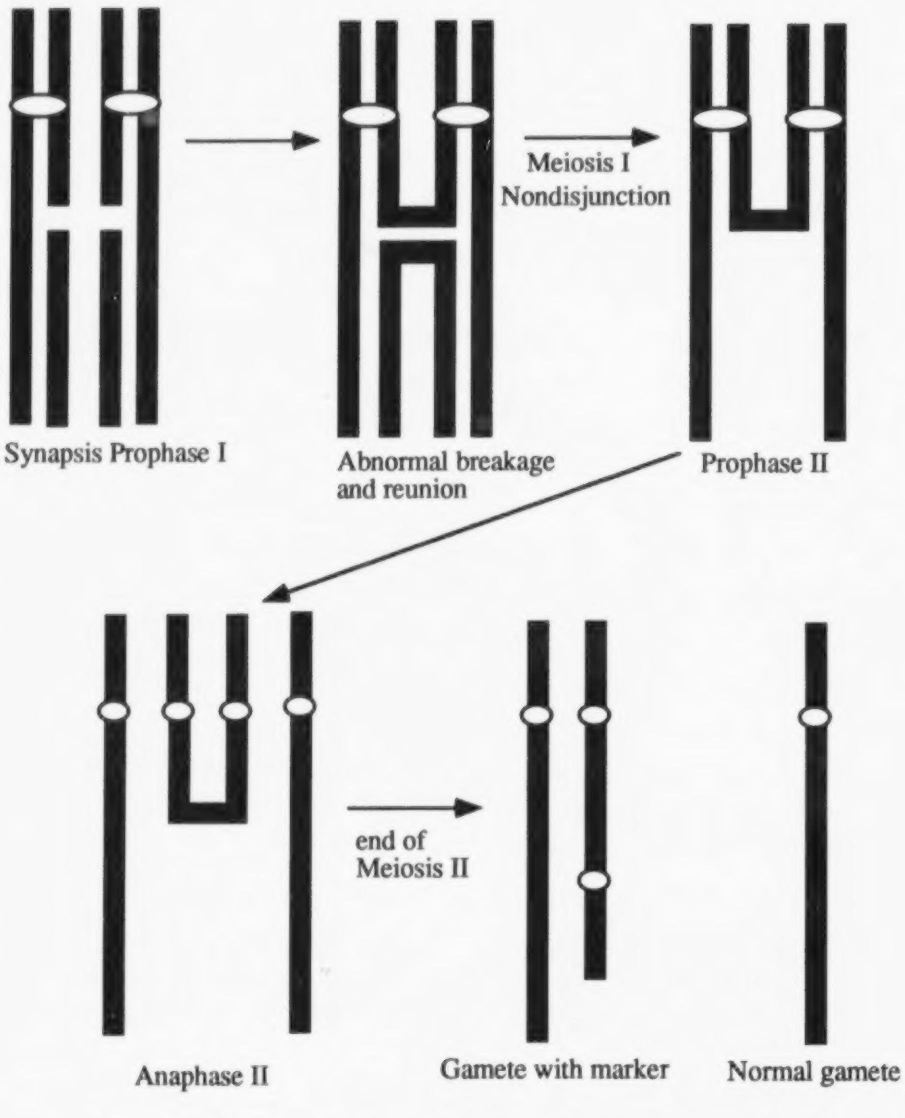

Synapsis Prophase I
Abnormal breakage and reunion
Meiosis I Nondisjunction
Prophase II
Anaphase II
end of Meiosis II
Gamete with marker
Normal gamete

Figure 22 Theoretical model of marker formation via abnormal breakage and reunion between homologous non-sister chromatids. Redrawn from Van Dyke et al. (1977)

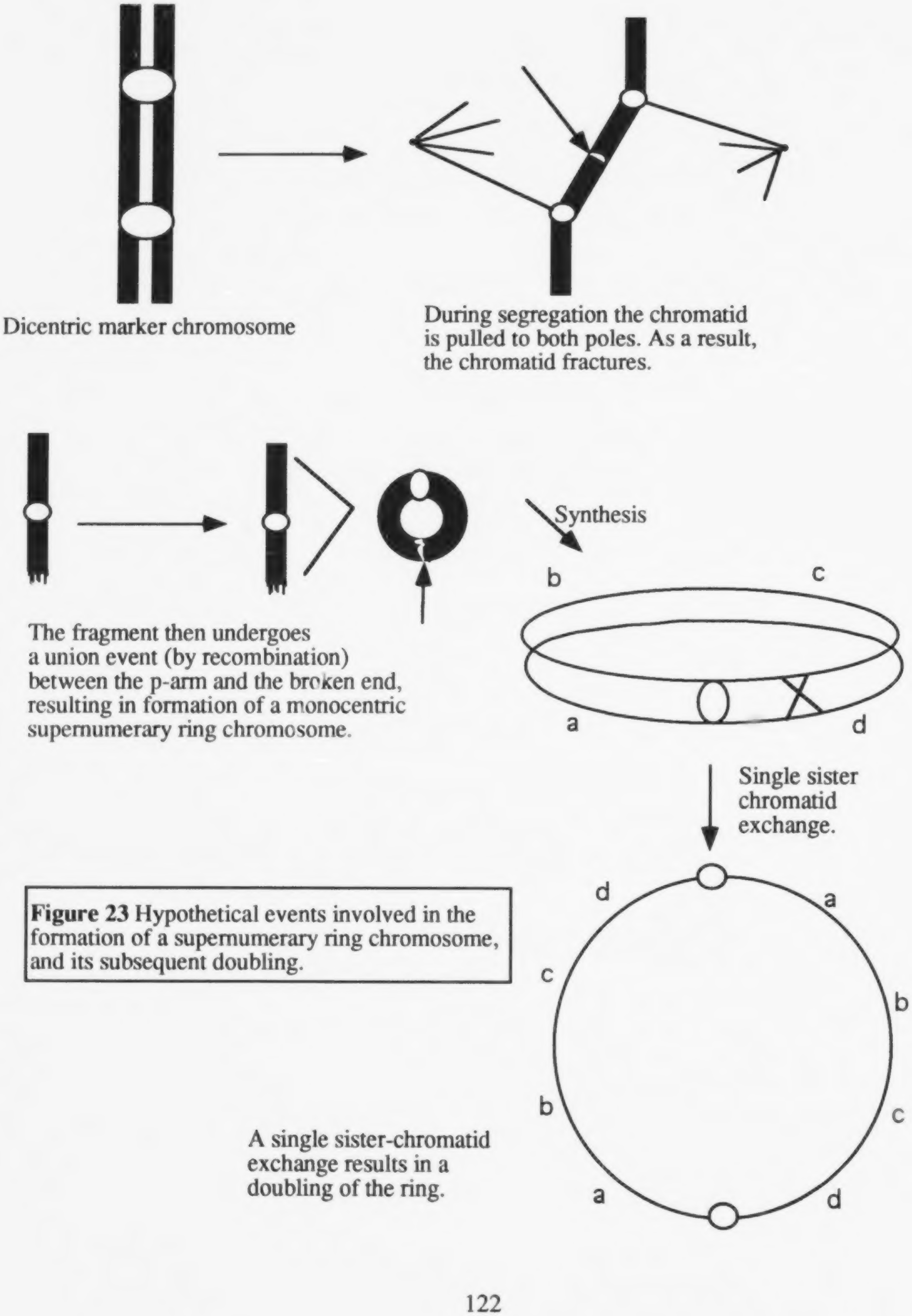
described with an extremely unstable marker chromosome. As a result of this instability, not only is mosaicism observed, but smaller derivative products are described. These included monosatellited fragments and, most interestingly, ring chromosomes. They assumed that breakage and unequal exchange of the original marker chromosome was responsible for these additional structures. It was suggested that some of the fragments may be a result of instability of the ring chromosomes. Such events that may have led to the formation of the ring chromosomes observed in CM13-15 and CM16 are shown in Figure 23. In this model, the dicentric product that has been formed by U-strand exchange for example, becomes unstable due to two active centromeres. As a result, it breaks at a random position between the centromeres. Depending on the position of the break, the monocentric products may be of a similar size or be very different. The next step involves the joining of the broken end of the q-arm and the short arm by a recombination event, which forms the ring.

In the family described in this study (CM13-15), the mechanism above is proposed to have originally produced the single ring chromosome structure observed in the grandfather (CM13) or an earlier ancestor. The unique breakpoint described for this ring chromosome may reflect a random break occurring between the centromeres of one of the marker chromosome types described in this study. The double ring that is observed in CM15 may have been formed by a sister chromatid exchange within the ring in the paternal germline, resulting in this larger dicentric ring (Figure 23). The ring chromosome of this family is somewhat unstable resulting in limited mosaicism and multiple rings in all individuals. CM16 is another example of a supernumerary ring chromosome 22, suggesting that such a process of formation is not vanishingly rare. The ring chromosome of CM16 is also unstable demonstrating more substantial mosaicism.

The ARRC22 Model

This hypothetical model is proposed on the basis of current molecular information on rearrangements of chromosome 22 and on the indirect evidence of the possible sequences involved in such rearrangements. This model assumes that the numerous repeat-rich regions along the long arm of chromosome 22 are responsible for the full spectrum of rearrangements.

The repeat-rich regions that are particularly prevalent in the 22q11-12 region may comprise low-copy repeats, Alu-elements, LINES or minisatellites. Each of these regions may vary in size, type of repeats, orientation of the repeats, and in the overall



Dicentric marker chromosome

During segregation the chromatid is pulled to both poles. As a result, the chromatid fractures.

The fragment then undergoes a union event (by recombination) between the p-arm and the broken end, resulting in formation of a monocentric supernumerary ring chromosome.

Single sister chromatid exchange.

Figure 23 Hypothetical events involved in the formation of a supernumerary ring chromosome, and its subsequent doubling.

A single sister-chromatid exchange results in a doubling of the ring.

structure of the region. The degree of similarity between the different repeat regions is assumed to determine which regions may be involved in misalignment and aberrant exchange during synapsis. The extent of the misalignment determines the size of the rearrangement, and the relative orientation of the repeats determines if these rearrangements are tandem or inverted. Due to the central role of the repeats in synapsis and aberrant exchange, this model is referred to as the ARRC22 model (Anchored Repeat Rearrangements of Chromosome 22). As shown in Figure 24, this model may be used to explain the different types of rearrangements associated with chromosome 22.

The formation of marker chromosomes relies on the inverted orientation of repeats of the two regions involved, with respect to each other. In order for the repeats to align in correct orientation, the chromosomes must align inverted with respect to each other (Figure 24D). Alternatively, physical distortion or looping of the proximal long arm may also result in the inverted alignment required. The determination of whether the markers are asymmetric or symmetric is based on which repeat-rich regions are involved. In this study, the majority of exchanges take place between the region flanked by the D22S181 and D22S36 loci. The prevalence of this site for most of the marker associated breakpoints may be an indication of the greater similarity (homology) of these repeat regions which produces the most common type of marker chromosome identified in this study (Type 1). In the case of the CM02 and CM03 marker chromosomes, the exchange takes place between the D22S181-D22S36 region and a more distal region (or regions, if the two markers differ). The marker of CM01 involves exchange between two distal regions currently unidentified. The latter three marker chromosomes, if considered part of a representative sample, are the products of less frequent exchanges. The extreme physical distortion or chromosomal inverted synapsis required for marker chromosome formation may be reflected by such rearrangements occurring at the lower end of the frequency spectrum.

The interstitial duplications of CE01 and CE02 would be the result of a large misalignment between the chromosomes 22, with the one repeat region being close to the centromere, the other much more distal (Figure 24C).

The DGS deletions vary considerably in size from cytogenetically detectable deletions (somewhat reciprocal to the interstitial duplications described above) to submicroscopic deletions. The most common deletions are in the 2 Mb size range. Halford et al. (1993a) have already suggested the presence of repeats flanking the DGS region which could be responsible for the unequal crossover and exchange resulting in deletion (Figure 24B). The presence of these deletions would suggest that reciprocal duplications may also occur. The fact that no such duplications have been reported may

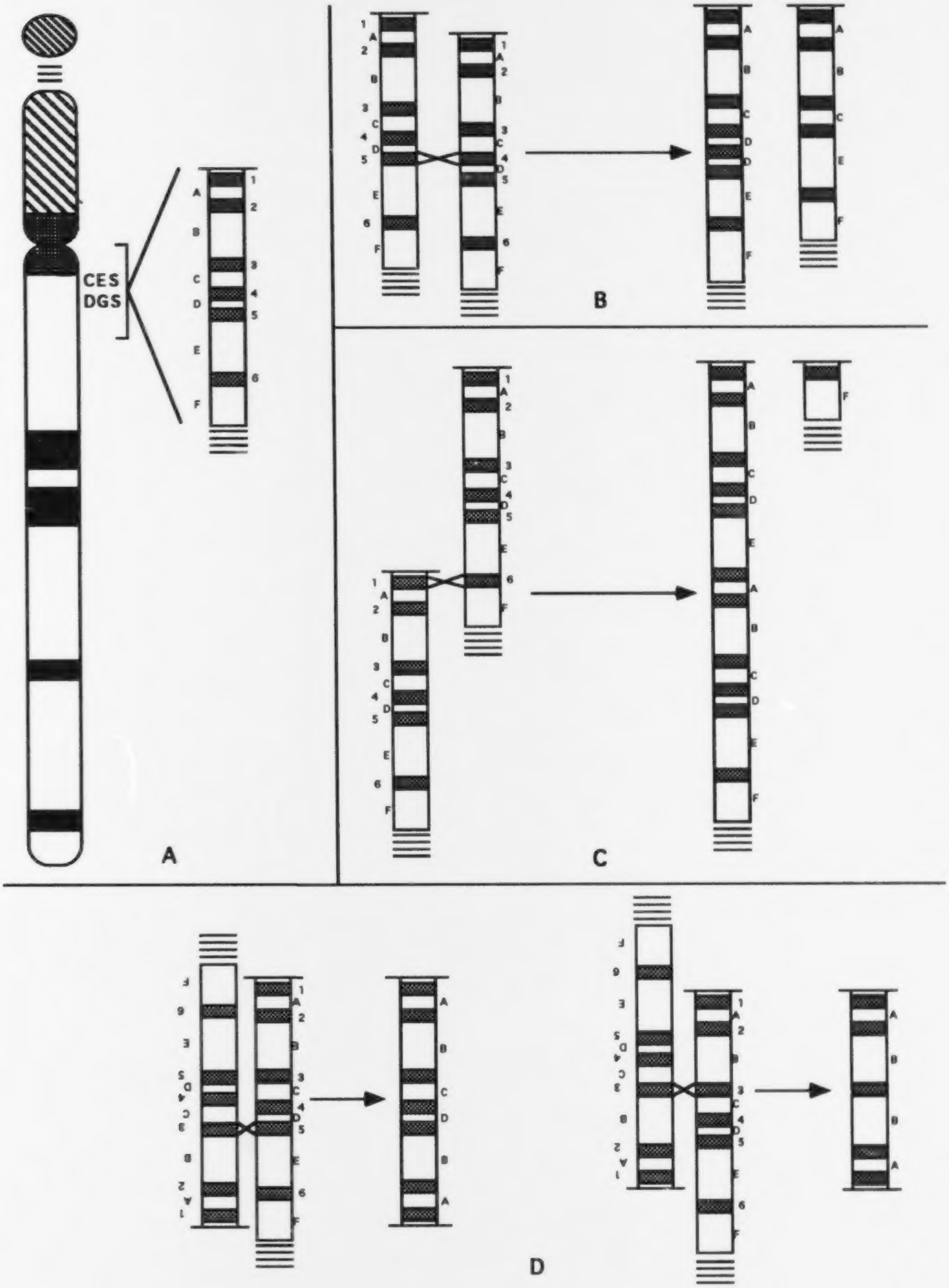
Figure 24 The ARRC22 model

In panel **A**, to the left is an ideogram of chromosome 22. The CES/DGS region is expanded into the diagramatic box to the right. The shaded numbered regions indicate the hypothetical repeat regions. The unshaded lettered regions represent the loci of 22q11.2. The horizontal bar above repeat #1 represents the centromere and p-arm. The horizontal bars at the bottom of the box represent the rest of the long arm.

Panel **B** demonstrates misalignment and exchange between two close repeat regions #4 and #5. The resulting products demonstrate a small interstitial duplication like that observed for D22S36 in this study. Exchange between more distant regions (#5 and #6, for example) may result in the larger interstitial deletions observed in DGS.

Panel **C** demonstrates a large misalignment and exchange between distant repeats #1 and #6. The resulting products represent large duplications and deletions of the region, as observed in the interstitial duplications of CE01 and CE02 in this study, and in some cases of DGS, respectively.

Panel **D** demonstrates the inverted alignment between the same repeat regions (right) and different repeat regions (left), resulting in symmetric and asymmetric marker chromosomes respectively.


CES
DGS
1
A
2
B
3
C
4
D
5
E
6
F
A
B
C
D

be due to the lack of any associated clinical phenotype, as preliminary data in this study have implied.

Small interstitial duplications have been identified in this study. The D22S36 duplications of CM04 and CM11 could result from a small misalignment and exchange between repeat regions mapping between D22S36-D22S181 and D22S36-D22S75. Of interest, one of the most common DGS deletions has a breakpoint mapping between D22S36-D22S75. The fact that these interstitial duplications were identified in 2/11 marker chromosomes of independent origin and in the father (YM11) of CM11, raises questions on the occurrence of this interstitial duplication. It is possible that the identification of these duplications in individuals with marker chromosomes is a mere coincidence, and this duplication occurs naturally at a relatively high frequency in the populace. A second possible explanation is that this interstitial duplication is somehow related, as either cause or effect, to the formation of the marker chromosome. If the duplication results in expansion of a repeat region, this may aid in the process of misalignment during synapsis. If this theory is correct, one might predict that the marker chromosome of CM11 is inherited paternally from YM11. Unfortunately, the inability to obtain a maternal sample stops definitive identification of the origins of this marker chromosome.

The ARRC22 model represents a unifying mechanism for the rearrangements of chromosome 22. Further examination of this model relies on the cloning of breakpoints associated with both DGS deletions and CES duplications.

Future Research

Identification of genes

The objective of studying CES is to discover the underlying molecular basis of the syndrome by identification of the genes involved. The first step for any aneuploid syndrome that spans a very large chromosomal segment, is to delineate, by phenotypic mapping, the smallest region to which many or all of the phenotypic traits (and associated genes) map. In this study, phenotypic mapping was required as little molecular information was known about the duplication associated with CES except it involved chromosomal band 22q11.2. From the analysis, the critical region has been mapped to a region spanning a maximum of 2.0 Mb. Within this region, defects associated with the heart, kidneys, genitals and facial dysmorphism are further mapped to a 1.0 Mb subregion.

Estimates for gene frequency in the human genome vary from one gene per 20 kb (gene-rich regions) to one gene per 200 kb (gene-poor regions) (Fields et al. 1994). This corresponds to 5-50 genes per megabase of DNA. Estimates of gene frequency for chromosome 22 suggest that chromosome 22 is relatively gene-rich (Scambler 1994). Recently, a YAC contig (Yeast Artificial Chromosome) has been produced that spans the complete CES region (McDermid et al., submitted). This resource will greatly aid in the future search for genes in the region. Two particularly successful techniques currently employed are exon trapping (Duyk et al. 1990) and direct cDNA selection with YACs (Lovett et al. 1991; Parimoo et al. 1993; Snell et al. 1993). Exon trapping is based on the identification of spliced exons within cloned genomic DNA in cosmids. Cosmids may be identified by screening of the appropriate cosmid library with labelled total YAC DNA. Direct cDNA selection with YACs involves the hybridization of labelled YACs (either membrane-bound or in solution) with cDNAs from a library (fetal brain). This technique has the advantage of isolating transcription products from very large regions in one step and has been used to great success (Peterson et al. 1994). The disadvantage of this technique is that it requires good representation of cDNAs in the library. Both techniques are viable means by which genes may be isolated from the CESCR.

To date, only one gene has been reported within the defined CESCR. ATP6E (Baud et al. 1993) encodes the 31kDa E-subunit of vacuolar ATPase. This gene is ubiquitously transcribed, and the enzyme comprising five subunits (A-E) plays a key role in controlling cellular environment. It is possible that increased dosage of this gene and its encoded subunit may disrupt the stoichiometric balance so as to disrupt enzyme function although the ubiquitous nature of the product makes it unlikely that overexpression would result in such specific defects as observed for CES.

At this point of gene identification, the next problem is how to test a gene to determine if it may be involved in the disease etiology. One method is provided by the resource of individuals with CES but normal karyotypes. If one assumes that these individuals possess mutations resulting in a CES phenotype, one may test candidate genes for possible dosage change or rearrangements by Southern blot analysis. It is interesting to note that in this study, no interstitial duplications were detected in such patients (CN01-03) for any of the tested loci including ATP6E. However, such a result does not rule out a potential role for any genes. As for the number of genes that may be involved, individuals have argued that for DGS, only a few or even one gene may be involved in disease etiology and this gene may have a key role in neural crest migration which would affect numerous tissues (Aubry et al. 1993). If such a mechanism was implicated for CES, single-gene defects may account for expression of CES in karyotypically normal individuals, but no theme like neural crest links all the CES features. A second means of testing a candidate gene is through the production of transgenic mice. As CES is associated with overexpression of genes, if syntenic regions to the CESCR or even homologous genes are identified in mouse, multiple copies of such sequences may be incorporated into transgenic mice (Constantini and Lacy 1981) to determine if overexpression causes any CES-like features.

Origins of the duplications

There is considerable interest in the mechanism by which the duplications of 22q11.2 are formed, especially with the inverted duplications of the marker chromosomes. Both chromosome 15 and 22 seem to possess certain unique properties that make them particularly unstable. Considerable information towards understanding such mechanisms may be elucidated by the cloning and sequencing of breakpoints. One apparent "hotspot" for such breakpoints in marker formation is the region between the D22S181 and D22S36 loci. Analysis would enable determination of whether the breakpoints cluster, if there are repeats involved and if the Type 1 marker chromosomes are truly symmetrical. Unfortunately, the region is large (maximum estimate is 1.1 Mb, McDermid et al., submitted) and there are considerable problems associated with cloning in this region (Marcia Budarf, personal communication) and as such may evade such analysis at present. Attention may well be directed towards more obtainable breakpoints that map in small and readily cloned regions. The small interstitial duplications of D22S36 may represent a more feasible target (CM04, YM11 and CM11). One of the breakpoints maps in the D22S36-D22S75 region, which spans only 250-300 kb. Furthermore, due to interest in this region stemming from DGS deletion analysis, this area is mostly cloned and sequenced (Marcia Budarf, personal communication), and hence may represent a rapidly achievable target. Identification of the one breakpoint may feasibly lead to isolation of the second breakpoint which maps distal to D22S36.

Besides the breakpoints that have been localized between two loci, there is also interest in the location of the distal breakpoints associated with the larger marker chromosomes and the interstitial duplications. It is not yet known how distal these breakpoints map and whether they map in proximity to each other or the breakpoints involved in translocations. If they are found to map to other "unstable" regions, then further evidence is provided for common mechanisms involved in the chromosome 22 rearrangements.

Due to the nature and origins of CES, identification of genes and analysis of the duplications and their breakpoints will provide considerable insight into the biology of the syndrome, and the structure and instability of chromosome 22.

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Appendix I

Quantitative Hybridization of Southern Blots

In order to determine copy number from quantitative hybridization, it is important to consider the variables and limiting factors associated with the methodology.

The major points of variability include:-

(1) Transfer by Southern blotting
(2) Hybridization and rehybridization after probe removal
(3) Background on the autoradiogram
(4) Quantification of the signal
(5) Linear range of the film

(1) Transfer variability

Transfer of DNA from an agarose gel to a nylon membrane was considered to be a relatively efficient and even process. Even thickness of the gel, removal of air bubbles and even weight distribution for the capillary process were all necessary to avoid transfer variability. One variable that was difficult to control was the different efficiences with which large (low) and small fragments (high) of DNA were transferred. Pretreatment by acid-nicking DNA with 0.25 M HCl increased the efficiency of transfer, but it was found that quantitative analysis using large fragment DNAs was more problematic and hence was avoided whenever possible.

(2) Hybridization and rehybridization

Hybridization refers to the annealing of radioactively labelled probe DNA, in the aqueous solution, to the target genomic DNA on the membrane. This process is a dynamic one and is subject to random chance variables. These may include variations of annealing efficiencies across the membrane and the variability of incorporation of radionucleotides in the population of probe DNA molecules. If DNA from a given sample was hybridized to two probes and their signals quantified and compared, the ratio (probe signal 1 / probe signal 2) should have been identical for each replicate, assuming no variability. The variability was studied by analyzing twenty data sets (seven ratios each) for 4 different test probes on two different blots. Variability was measured as the percent deviation (difference) from the mean determined for each of the data sets. No significant differences were observed between blots or probes. With all 140 deviation values (%) combined it was found that 65% of the ratios fell within a 10% deviation from the mean, 90% fell within a 20% deviation from the mean, and 98% fell within a 25% deviation.

The same variable was measured to determine if the stripping of blots (removal of bound probe) and rehybridization to new probes increased the variability of the data. In order to study these effects a blot was probed, signals quantified from it, stripped, then rehybridized with the same probe (to avoid transfer variability associated with fragment size). The resulting 35 ratios (signal from probe in 1st hybridization / signal from probe in 2nd hybridization) showed a comparable distribution to the previous data determined for hybridization variability. This result suggested that rehybridization per se, was not responsible for a significant increase in variability of hybridization data.

(3) Background

The ideal situation for quantification of signals from autoradiograms is that each probe produces a clean, single band. Unfortunately, human genomic DNA probes often contain either repetitive sequence or sequence homologous to other regions of the genome besides the predicted target sequence. The signal to background ratio for a given probe can be optimized by preannealing to total DNA, but even this approach does not always eliminate the problem. Homologous bands in general were not a problem, and as such could simply be avoided when measuring signals. Background smears within the lanes needed to be subtracted from the primary signal to be measured. This background provided another source for variation when measuring bands (signals) on autoradiograms.

(4) Quantification

The signal was measured in the form of the area beneath the peak (integration) as determined from the quantification of optical density across the film (profile). The band would appear as a peak above the background optical density of the film and any background signal. The quantification of the peak required removal of the background, and this was the only part of analysis that required human judgement. Such a determination therefore was error prone. Repeated measures of single bands determined that a single person judgement resulted in a maximum of 5% variability in the quantification of a signal. This variation therefore could not account for all the variability of hybridization previously mentioned.

(5) Linear range of the film

One critical limitation associated with quantification from autoradiograms is the linear range of the film. As the signal increases, so does the optical density of the band, and this is reflected in the resulting measurement of that band. Within the linear range, if the signal is doubled, the maximum optical density (max OD) or peak height of the band is doubled, and the area beneath the peak as measured from the profile (OD/mm) should also double. As the film becomes saturated, and the OD limits of the film are reached, the resulting quantification of the band/peak is no longer proportional to the total signal. In order to determine the linear range of the film, a blot with increasing concentrations of DNA (0.2-25.6 μg) was hybridized with the probe to the D22S39 locus. The resulting autoradiograms were quantified and plots made of (i) max OD vs DNA amount, and (ii) area (sum density) vs DNA amount. The resulting graphs are shown in Figure 25 for the GS670 BioRad densitometer system. For the GS670 and the Kodak XAR-5 film, the linear range for maximum OD is 0.10-0.80, and for area 0.20-2.00. The Gelprint 2000i system demonstrated equivalent linear ranges (measured in greyscale pixels). The quantified bands were always measured within this linear range, so they accurately reflected the signal from the blot.

No obvious lower limit of linearity was detectable with these systems, although it is likely that the insensitivity of the densitometers in measuring distinct peaks above background levels for weak signals precluded the identification of any such lower limit. Preflashing was not used, as the benefits of such a technique are only required for weak signals, which were not produced by the methods described in this study. Furthermore, preflashing would increase the background level which would be detrimental to the quantification of autoradiographic signals.

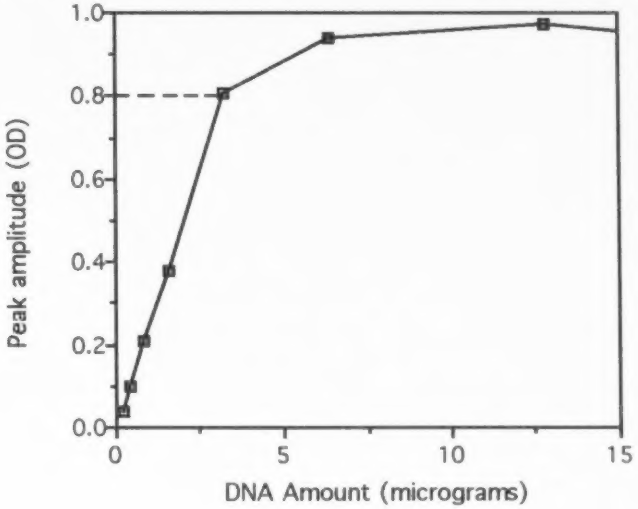
In general, quantitative hybridization can be a relatively accurate and reliable method of dosage analysis, but considerable care needs to be taken to ensure that the most accurate data is obtained and carefully analyzed.

Figure 25 Graphs demonstrating the linear range of the Kodak XAR-5 film used in autoradiographic quantification, using the GS670 BioRad scanning densitometer.

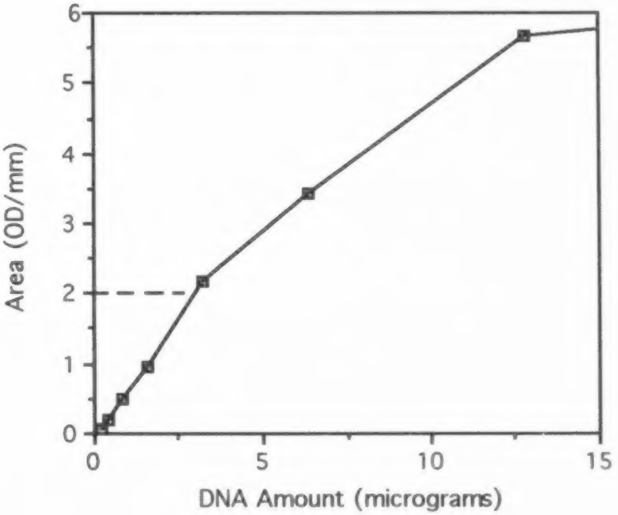
Graph (i) is a plot of the maximum optical density (peak amplitude measured from an autoradiographic band with the GS670 system) against DNA amount (in micrograms). The dotted line demonstrates the approximate end of the linear relationship which corresponds to a value of 0.80 OD.

Graph (ii) is a plot of the sum density (measured as the area under the profile curve (integral) as determined by the GS670 system) against DNA amount (in micrograms). The dotted line demonstrates the approximate end of the linear relationship and corresponds to a value of approximately 2.00 OD/mm.

(i) Plot of maximum optical density (amplitude) against DNA amount



(ii) Plot of peak area (OD/mm) against DNA amount



Appendix II

Statistical Analysis

Once hybridization data is obtained, it is important to analyze it in the context of its potential associated errors. Several methods have been used in the literature to analyze sets of standardized ratios. These methods have varied from simple eye judgement to complex regressional analysis. Depending on the level of dosage (number of copies) and the accuracy required, care should be taken in the methods of analysis. One of the commonly used methods of dosage analysis involves replicates of the test DNAs (1-3) being compared to the same number of replicates of control DNAs. The comparison is made between the means of these data sets. Typically the means of the controls are determined and the average of these two is chosen as the arbitrary cut-off point (*acp*). Any mean test value that falls below the *acp* is the lower copy value, any test value above represents the upper copy value (McGinniss et al. 1992). Considering the hybridization variables, this analysis is very simplistic and error prone. Although the standardized ratios typically fall within a small distribution (% deviation from the mean) for a given group, a single highly variable value may have a significant effect on the mean, especially on a small sample size. Furthermore, an arbitrary cut-off point ensures an answer, even when the data is so variable that it should be considered inconclusive. A judgement of inconclusive data may be made with this methodology, but again it would be an arbitrary decision.

Parametric tests such as the T-test provide a non-arbitrary method of analysis and give the level of confidence on the data and the resulting conclusions. Such methods assume a normal distribution of data. However, due to the sample sizes typically used for quantitative hybridization, the associated variables, and the requirements for a specific distribution of data, the stringent parametric test is inappropriate. The slot blot analysis method devised by Blouin et al. (1990) described a regressional analysis of large data sets. Basically, a gradient of 10 DNA aliquots from each individual sample were transferred to nylon membrane via a slot blot manifold. The nylon membrane was hybridized to the reference probe, stripped, then rehybridized to the test probe. For each sample, a line of 10 points was plotted, each point represented by the test probe signal (Y value) and its corresponding reference probe signal (X value). The resulting line plots were "forced" through the origin and then the slopes statistically compared. Though the test is relatively stringent and gives "pleasing" figures, preliminary analysis using this method discovered several flaws in the system. Firstly, the stringency of the test often excluded much of the data (in excess of 30%). Secondly, the conclusions were not reproducible. Thirdly, and most significantly, due to the use of a slot blot method of transfer, background signals could not be distinguished from the locus-specific signal. As discussed in Appendix 1, failure to eliminate background variability is a significant source of error, especially for probes producing a lot of background hybridization.

In the study described in this thesis, the Wilcoxon Rank sum test was chosen as a nonparametric means of analyzing data. It is sufficiently stringent, but requires no assumptions on the distribution of the hybridization data. In essence, the test determines whether two populations (data sets) are the same. This test provided a means of non-arbitrary testing which would recognize data that was inconclusive. Furthermore, as with all analysis of data sets, it was determined that a minimum of 3 (for RFLP) and typically 4-7 replicates would be used for each test, to ensure greater accuracy and stringency for the statistical analysis.

To outline the Wilcoxon Rank Sum test, an example will be used (actual data from one study). Note that copy number determination in this study was in the 2-4 copy range. With the variables associated with quantitative hybridization, large data sets of standardized ratios plotted as a distribution curve, will show overlap. Assuming the general distribution described in Appendix 1 for standardized ratios (large population), if a mean of ratio of 2.0 is assumed for a two-copy sample and 3.0 for the three-copy sample, nearly 10% of two-copy ratios will fall in the range of 2.3-2.6, and an equivalent 10% of three-copy ratios will also fall in the 2.3-2.6 range. Therefore, some overlap is predicted between data sets, but a large number of replicates reduces this effect and makes data more likely to be significant and less error-prone. Conversely, the problem with using only 1-3 replicates is very apparent, and could easily lead to erroneous conclusions.

The following data was obtained by calculating standardized ratios (test probe signal for N25/reference probe signal for p21-4U) for the two-copy (2) and three-copy controls (3) and two patients (CM04) and (CM03).

(2)	data (1.56, 1.57, 1.61, 1.70, 1.76, 1.81, 1.99)
(3)	data (1.80, 1.93, 2.06, 2.20, 2.25, 2.33, 2.39)
(CM04)	data (1.32, 1.48, 1.61, 1.63, 1.66, 1.77, 1.79)
(CM03)	data (1.90, 1.91, 1.99, 2.05, 2.16, 2.37, 2.43)

By general observation, the degree of overlap of data gives an idea of copy number. Two data sets are compared at a time. First of all, (3) is compared to (2), to determine that the differences in copy number are distinguishable.

Data values for the two sets are grouped and ranked from smallest to largest (1 to 14) with the data set (2) values shown in brackets . Then the rank values are added for each of the two groups and compared, hence resulting in a sum rank value.

Ratio	Rank	Sum of Ranks for (2)	Sum of Ranks for (3)
(1.56)	1	1	
(1.57)	2	2	
(1.61)	3	3	
(1.70)	4	4	
(1.76)	5	5	
1.80	6		6
(1.81)	7	7	
1.93	8		8
(1.99)	9	9	
2.06	10		10
2.20	11		11
2.25	12		12
2.33	13		13
2.39	14		14
Sum of Ranks	105	= 31	= 74

There is a slight overlap but to determine if the two data sets are significantly different, the sum ranks (SR) are compared in the appropriate statistical tables. The P-values (shaded) for the sum of ranks when comparing two data sets of 7 values each is shown below (Wilcoxon and Wilcox 1964):-

0.001	0.005	0.010	0.025	0.050	0.100
29	32	34	36	39	41

The hypothesis (one-tailed) is that the (3) data set is signficantly greater than the (2) data set. The SR (2) value of 31 showed that this data set was significantly less than the (3) data set, at a probability level of P=0.005 with P=0.05 as the level of significance. The patient data sets were then compared with the data sets of the controls with SR values being obtained as before. The results are shown for patients CM04 and CM03 comparing the copy number of the N25 probe (D22S75 locus).

Tested data sets	CM04	(2)	CM04	(3)
Sum of Ranks	46.5	58.5	28	77
Probability (P)	>0.10		0.001	
Conclusion	2 copies			

Tested data sets	CM03	(2)	CM03	(3)
Sum of Ranks	74.5	30.5	50	55
Probability (P)	0.005		>0.10	
Conclusion	3 copies			

This example demonstrates how the Wilcoxon Rank Sum test is performed. It is important to note that the test does not always result in conclusive data and in such cases, results such as 2/3 (2 or 3 copies) is possible i.e. patient data set is significantly greater than disomic control data set but significantly less than trisomic control data set.